이학박사학위논문

P19 세포의 분화과정에서 Centrobin과 CEP215의 미세소관 형성 유도 기능에 관한 연구

Studies on cytoplasmic microtubule organizing activity of Centrobin and CEP215 during glial cell differentiation of P19 cells

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Abstract

Studies on cytoplasmic microtubule organizing activity of Centrobin and CEP215 during glial cell differentiation

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Microtubule is a cytoskeleton which plays a role in cell morphology and intracellular transport. In mitotic cells, microtubules become spindles to pull a set of chromosomes into daughter cells. The centrosome is the principal organizer of microtubules in most animal cells. However, microtubule organization outside the centrosome is also observed in specialized cells, such as neurons and polarized epithelial cells. The γ-Tubulin ring complex (γ-TuRC) is a key structure for microtubule organization. Subcellular localization and activity of γ-TuRC should be tightly regulated in accord to physiological status of the cell. Here, I studied two centrosomal proteins, centrobin and CEP215, which are essential for microtubule organization outside the centrosome.

In chapter 1, I observed that specific cytoplasmic centrobin is associated with stable microtubules. Centrobin is a daughter centriole-specific protein which is critical

for centriole duplication. In hippocampal cells, centrobin formed cytoplasmic dots in addition to the localization at both centrosomes with the mother and daughter centrioles. Such specific localization pattern suggests that cytoplasmic centrobin is not just a reserved pool for centrosomal localization but also has a specific role in the cytoplasm. In fact, centrobin enhanced microtubule formation outside as well as inside the centrosome. These results propose specific roles of the cytoplasmic centrobin for noncentrosomal microtubule formation in specific cell types and during the cell cycle.

In chapter 2, I observed specific localization of CEP215 along the processes of astrocytes in cultured mouse hippocampal cells and differentiated P19 embryonic carcinoma cells. GFAP expression and process formation were suppressed in *CEP215*-deleted P19 cells. The phenotypes of *CEP215* deletion were rescued by ectopic Flag-CEP215, but not by Flag-CEP215$^{\Delta PCNT}$ and Flag-CEP215^{F75A}, which do not interact with pericentrin and γ-tubulin, respectively. I observed reduction of the centrosomal γ-tubulin levels in *CEP215*-deleted P19 cells. Based on the results, I propose that the microtubule nucleating function of CEP215 is essential for glial differentiation.

Taken together, these results showed the importance of microtubule organizing function of centrobin and CEP215 outside the centrosome. My work is the first report of a centrosomal protein for gliogenesis and process formation.

Keywords: Microtubule nucleation, centrobin, NEK2, CEP215, gliogenesis, process formation, PCM

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Background

1. Centrosome

Centrosome is the major microtubule organizing center (MTOC) of most animal cells. In the past, centrosome study focused mainly on regulation of cellular morphology or migration related to microtubule nucleating activity of centrosome during interphase. Through the past study, various centrosomal proteins and their regulation mechanism were revealed. Now, studies about centrosomal proteins are not confined to centrosome, and they also perform important functions outside the centrosome. Recently, centrosome study broadened its scope to aneuploidy and cancer, asymmetric division of progenitor cells and cilia formation. It has been found that the function of centrosome is related to various disease such as microcephaly, dwarfism and ciliopathy.

1.1. Structure of centrosome

Centrosome consists of two centrioles which are the base of the centrosome. The centrosome is also duplicated only once per cell cycle like chromosome, and two centrioles can be divided into mother and daughter centriole according to the duplicated period. Two centrioles are surrounded by pericentriolar material (PCM), and PCM proteins recruit γ-tubulin ring complex (γ-TuRC) components for microtubule nucleating activity of centrosome (Conduit et al., 2015) (Figure 1).

1.2. Functions of centrosome

The major functions of centrosome can be divided by cell cycle. As a major cellular MOTC, centrosome nucleates microtubule network and maintains the cellular cytoskeleton during interphase. During mitosis, centrosome functions as mitotic spindle pole and organizes mitotic spindle by recruiting PCM proteins. Therefore, centrosome can regulate chromosome segregation and cell division (Fukasawa, 2007). The mother centriole of the centrosome functions as basal body of cilia during quiescent phase, and is involved in formation of cilia and command various cell signaling deliver (Nigg and Raff, 2009) (Figure 2).

2. Microtubule nucleation

Microtubule is one of the essential cytoskeleton in the cell and supports the cellular morphology. The organization of microtubule network in the cell determines area or morphology, and regulates cellular migration. The strong microtubule nucleation from spindle pole during mitosis regulates chromosome segregation and actually cell division. Recently, many factors that regulate microtubule nucleation have been revealed. Most of them are components of γ-TuRC, centrosomal proteins that recruit γ-TuRC components and microtubule associated proteins (MAP). These proteins regulate microtubule nucleation both at the centrosome and the cytoplasm, and finally decides the morphology of the cell.

2.1. Microtubule nucleation from γ-TuRC

The microtubule nucleation primarily depends on γ-tubulin and γ-TuRC components which are associated partners of γ-tubulin (Lin et al., 2015). The γ-TuRC components containing γ-tubulin function as scaffolds for α/β-tubulin dimers to initiate polymerization (Lin et al., 2015). Microtubule is majorly nucleated from centrosome, because γ-TuRC components are recruited by PCM proteins. The representative PCM proteins that recruit γ-TuRC components to the centrosome are pericentrin and CEP215 (Figure 3).

2.2. Cytoplasmic microtubule nucleation

The microtubule nucleation can be occurred outside the centrosome such as Golgi and chromosome. Even the microtubule itself can be a template for microtubule nucleation. These microtubule nucleation are also mostly mediated by γ-TuRC components. For example, PCM protein CEP215 can induce microtubule nucleation not only at the centrosome, but also in the cytoplasm and *in vitro* by direct interaction with γ-TuRC components (Choi et al., 2010). Golgi can function as MTOC because PCM proteins such as CEP215 and AKAP450 are concentrated at Golgi and recruit γ-TuRC components for microtubule nucleation. The noncentrosomal microtubule can be generated by release from the centrosome, nucleation at noncentrosomal sites and breakage of pre-existing microtubules mediated by MAP proteins (Bartolini and Gundersen, 2006).

3. Neuronal cell morphology

Neuronal cells show specific asymmetric morphology consist of diverse types of neurites such as axon and dendrite. The neurites are supported by strong cytoskeleton such as highly stable acetylated tubulins. Therefore, microtubule nucleation and factors regulate microtubule network decide the morphology of neuronal cells. Recently, it is revealed that the major cellular MOTC centrosome and some centrosomal proteins can determine neuronal morphology by regulating microtubule.

3.1. Microtubule network of neuronal cells

The polarity of microtubule polymerization is determined by γ-tubulin mediated microtubule nucleation during neurite formation (Delandre et al., 2016). Therefore, anterograde polymerization is dominant in neuronal cells forming neurites actively. The polarity of microtubule polymerization is one of the important factor in neurite formation (Baas and Lin, 2011) (Figure 4). In the case of neurons, Golgi fragments localize at the branching point of neurites, and the neurite spreads out through anterograde polymerization from Golgi fragments (Ori-McKenney et al., 2012). Recently, it was revealed that centrosomal proteins such as γ-tubulin, AKAP450 and CEP215 are important for neurite formation using their microtubule nucleation activity related to above mechanism. Actually, these proteins also regulate neurite formation and overall morphology of neuronal cells (Yalgin et al., 2015).

3.2. Morphology of neuronal cells

The morphology of neuronal cells is determined by polarity and formation of neurites mediated by microtubule nucleation. The PCM proteins can induce microtubule nucleation by recruiting γ-TuRC components, and CEP215 can recruit γ-TuRC components not only to centrosome, but also to Golgi or cytoplasm (Wang et al., 2010). In post mitotic neuronal cells, the centrosome does not function as major MTOC, and microtubule nucleation occurs at acentrosomal sites (Stiess et al., 2010). For example, in *Drosophila* neuronal cells, AKAP450 recruits γ-TuRC components to Golgi and determines polarity of microtubule nucleation, and finally decides the neurite formation and morphology (Lewis and Polleux, 2012). The PCM protein CEP215 regulates branching of neurons, and it was recently reported that CEP215 also regulates dendrite branching of *Drosophila* da (dendritic arborization) neurons (Yalgin et al., 2015). Therefore neuronal morphology can be determined by some centrosomal proteins which are involved in microtubule nucleation.

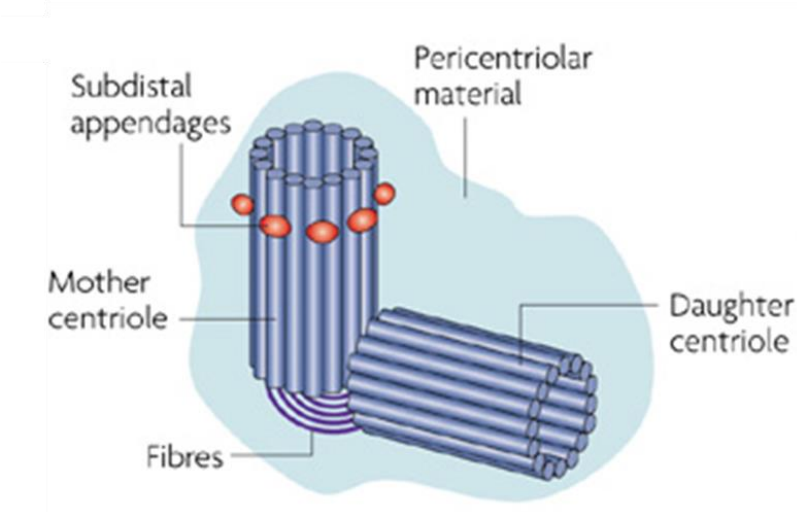


Figure 1. Structure of centrosome. The basic structure of centrosome is mother centriole, daughter centriole and PCM (Fukasawa, 2007).

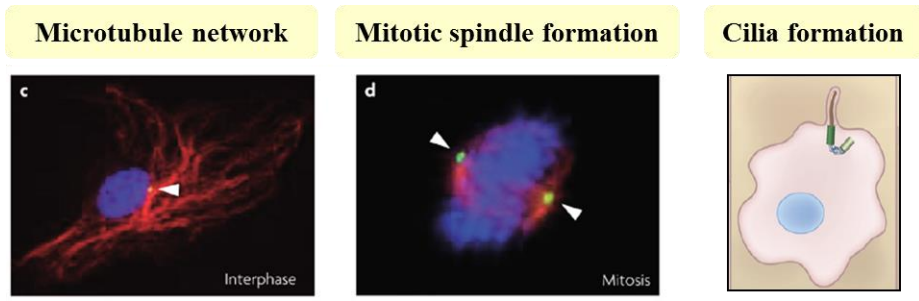


Figure 2. Functions of centrosome. The centrosome functions change during cell cycle. The centrosome function as MTOC and involved in formation of spindle pole during mitosis. Mother centriole is served as basal body for cilia formation (Fukasawa, 2007; Nigg and Raff, 2009).

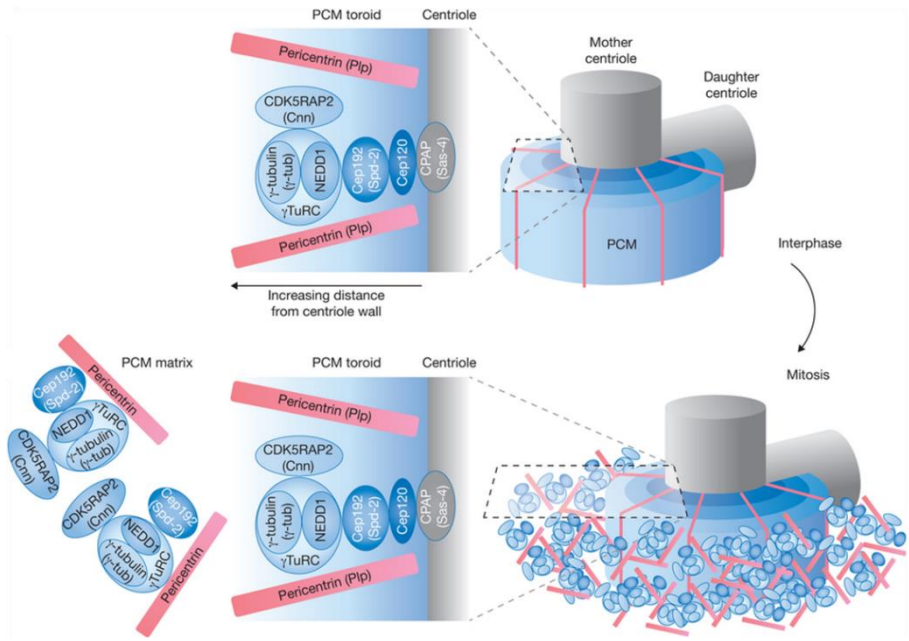


Figure 3. Recruitment of γ-TuRC components to centrosome by PCM. The γ-TuRC components are recruited to the centrosome by PCM proteins such as pericentrin and CEP215/CDK5RAP2 (Luders, 2012).

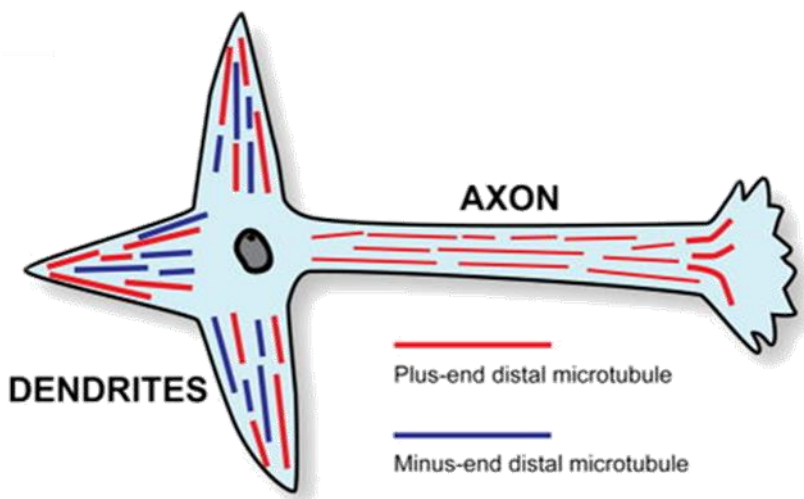


Figure 4. Formation of neurite by polarity of microtubule polymerization. The anterograde polymerization is dominant where the neurites form (Baas and Lin, 2011).

Chapter 1.
The microtubule nucleation activity of centrobin in both the centrosome and cytoplasm

Abstract

Centrobin is a daughter centriole-specific protein which is critical for centriole duplication. Nucleation and stabilization of microtubules are known biological activities of centrobin. Here, I report a specific localization of centrobin outside the centrosome. Centrobin was associated with the stable microtubules. In hippocampal cells, centrobin formed cytoplasmic dots in addition to the localization at both centrosomes with the mother and daughter centrioles. Such specific localization pattern suggests that cytoplasmic centrobin is not just a reserved pool for centrosomal localization but also has a specific role in the cytoplasm. In fact, centrobin enhanced microtubule formation outside as well as inside the centrosome. These results propose specific roles of the cytoplasmic centrobin for noncentrosomal microtubule formation in specific cell types and during the cell cycle.

Introduction

Microtubules may be assembled and disassembled without an enzyme activity, which is best described with the dynamic instability model (Mitchison and Kirschner, 1984). In reality, however, the nucleation and stability of microtubules in live cells are largely controlled by protein factors. A number of microtubule-associated proteins have been identified to have a nucleating and stabilizing activity of microtubules in vitro. For example, doublecortin enhances microtubule nucleation and stabilization with a specific interaction with protofilaments (Bechstedt and Brouhard, 2012; Moores et al., 2004).

Microtubules of animal cells are strongly influenced by the centrosome where the minus ends of microtubules are concentrated. Meanwhile, a fraction of microtubules may be nucleated outside the centrosomes, especially in neuronal, epithelial and muscle cells. The noncentrosomal microtubules may be generated de novo or transported from the centrosome to the site (Bartolini and Gundersen, 2006). A list of microtubule-associated factors for the noncentrosomal, cytoplasmic microtubules has been identified (Cassimeris and Spittle, 2001).

Centrobin was initially known as a daughter centriole-specific protein which is required for centriole duplication and elongation (Zou et al., 2005). Centrobin binds to microtubules and stabilizes them (Gudi et al., 2011; Jeong et al., 2007; Lee et al., 2010). Two different kinases are known to regulate the microtubule stabilizing activity of centrobin in opposite ways. PLK1 enhances the microtubule stabilizing activity of centrobin during mitosis whereas NEK2 antagonizes it in interphase cell (Lee et al.,

2010; Park and Rhee, 2013). The microtubule organizing activity of centrobin is critical for asymmetric division of *Drosophila* neuroblasts (Januschke et al., 2011). Centrobin is necessary and sufficient for daughter centriole anchoring at the apical region of the neuroblasts (Januschke et al., 2013).

Studies on the biological activity of centrobin have been limited to the centrosome. In this study, I expand biological function of centrobin to the cytoplasm. I determined subcellular localization of the cytoplasmic centrobin. Furthermore, I revealed that the cytoplasmic centrobin is essential for noncentrosomal microtubule formation.

Materials and Methods

Antibodies

The centrobin antibody was previously described (Jeong et al., 2007). The polyclonal and monoclonal α-tubulin antibodies were purchased from Abcam and Sigma, respectively. The antibodies against centrin2, γ-tubulin, GAPDH and GFP were purchased from Millipore, Santa Cruz, Ambion and Santa Cruz, respectively. The Alexa-fluorescence secondary antibodies were purchased from Invitrogen.

Cell culture

The U2OS cells were cultured in McCoy's 5A media supplemented with 10% FBS. The plasmid DNA was transfected in U2OS cells using Fugene HD (Promega) according to the manufacturer's instruction. The Tet-on stable U2OS cell lines were generated using 400μg/ml of G418 (Calbiochem) selection. The TRE double stable U2OS cell lines were generated with hygromycin B (Clontech) according to the manufacturer's instruction.

The mouse hippocampus cells were cultured following a general protocol with modifications (Kaech and Banker, 2006). Hippocampi were extracted from E17 mouse embryos and dissociated mechanically after trypsin treatment. The cells were washed with HBSS, and then 250,000 cells/cm2 were plated on the poly-D-lysine coated coverslips in plating media (10% FBS, 0.45% glucose, 0.1mg/mL sodium pyruvate in 2mM glutamine-containing MEM/EBSS). Cells were cultured in the neurobasal media supplemented with B27, glutamax and penicillin/streptomycin after 3 h of recovery.

Plasmids and RNA interference

The wild type and mutant centrobin cDNA were subcloned into pTRE2hyg-GFP. siRNAs specific to centrobin (5'-GGATGGTTCTAAGCATATC-3'), 5'-UTR of centrobin (5'-GGACTTTGCTAAAGCAGAA-3'), and NEK2 (5'-GGCAAATTCAGGCGAATTC-3') were purchased from ST Pharm, and were transfected using RNAiMAX (Invitrogen) according to the manufacturer's instruction. Non-specific control siRNA (5'-GCAATCGAAGCTCGGCTAC-3') was used.

Immunoblot analysis

The protein samples for immunoblot were lysed using sodium dodecyl sulfate sample buffer, and then subjected to SDS-polyacrylamide gel electrophoresis. The gel was transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 0.1% TBST (Tris-buffered saline TBS with 0.1% Tween 20) for 1 h. The membrane was incubated with primary antibody overnight at 4˚C, and then washed with 0.1% TBST 3 times. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature, and then washed with 0.1% TBST 3 times. The ECL solution was treated onto the membrane, and then exposed to an X-ray film.

Immunocytochemistry, image processing and statistical analysis

The cells on the coverslip were fixed with methanol for 10 min and washed with phosphate-buffered saline (PBS) 3 times. The fixed cells were blocked with 3% bovine serum albumin in 0.5% PBST (PBS with 0.5% Triton X-100) for 20 min, incubated with the primary antibody for 1 h, washed with 0.1% PBST 3 times, incubated with the secondary antibody for 30 min, and then washed with 0.1% PBST 3 times. The coverslips were mounted on a slide glass using Prolong gold mounting solution (Invitrogen) after DAPI incubation. The immunostained cells were observed using fluorescence microscope with a CCD (Qi-cam Fast 1394; Qimaging) camera and processed with ImagePro 5.0 (Media Cybernetics, Inc.) and Adobe Photoshop software. The length of microtubule was measured using ImagePro 5.0 2(Media Cybernetics, Inc.). The intensity of centrosomal microtubule was measured by drawing the circle including the nucleated microtubules, and background was excluded by drawing same size circle in the nearest cytoplasm. All distinguished centrosomal and cytoplasmic microtubules were counted per cells for quantifying the centrosomal and cytoplasmic microtubules. For statistical analysis, n=90 per group in three independent experiments. The statistical significance was determined by Student's t-test using SigmaPlot (Systat Software, Inc.).

Microtubule regrowth assay

The microtubule regrowth assay was conducted following an optimized method for observing cytoplasmic microtubules (Choi et al., 2010). To disrupt microtubule network, U2OS cells were placed on the ice for up to 90 min. To induce microtubule regrowth, the cells were incubated with warmed McCoy's 5A media for indicated time periods, and immediately fixed with 4% paraformaldehyde in PEM buffer (80mM PIPES pH6.9, 1mM MgCl2, 5mM EGTA, 0.5% Triton X-100) for 10 min. The fixed cells were incubated with 0.3% PBST for 5 minutes to increase permeability.

Results

Centrobin is essential for microtubule nucleation at both the centrosome and cytoplasm

I performed immunocytochemical analysis to determine cytoplasmic distribution of centrobin in U2OS cells. The cytoplasmic signals of centrobin became visible when most of microtubules except the stable ones were disassembled at a low temperature. The cytoplasmic centrobin was detected along with the stable microtubules (Figure 5). This result is consistent with the previous report in which ectopic centrobin was colocalized with the bundles of stable microtubules (Jeong et al., 2007).

To study involvement of centrobin in microtubule assembly, I performed microtubule regrowth assays with centrobin-depleted U2OS cells. I depleted endogenous centrobin with siRNA transfection into U2OS cells (Figure 6A, B). The results showed that the intensity and the number of centrosomal microtubules were reduced in centrobin-depleted cells (Figure 7A-C). The number of cytoplasmic microtubules was also reduced in the centrobin-depleted cells (Figure 8). However, an average length of the centrosomal microtubules was not affected with the centrobin depletion (Figure 7D). These results suggest that centrobin is essential for microtubule nucleation in both the centrosome and cytoplasm, but not for microtubule elongation. I also observed microtubule regrowth for extended time periods up to 10 minutes to determine involvement of centrobin in microtubule anchoring (Delgehyr et al., 2005). The results showed that microtubule networks were reduced and disorganized in the

centrobin-depleted cells (Figure 9A, C). However, no significant difference in the centrosomal microtubules was observed between the control and centrobin-depleted cells, suggesting that centrobin may not affect the microtubule anchoring (Figure 9B) (Delgehyr et al., 2005).

C-terminus is required for microtubule nucleating activity of centrobin

The C-terminal half of centrobin includes a domain for the microtubule stabilizing activity (Jeong et al., 2007). In fact, a domain at the 765-903 residues is responsible for interaction with tubulin and for localization to the centrosome (Gudi et al., 2011). I generated inducible U2OS cell lines in which the wild type (GFP-CBN) and a C-terminal end-truncated mutant (GFP-CBN$^{\Delta C}$) of centrobin were expressed. I carefully adjusted the ectopic centrobin levels comparable to the endogenous levels using the leaky expression of ectopic GFP-CBN without doxycycline treatment (Figure 10A). An siRNA specific to centrobin 5'UTR (*siCBN*) was transfected to deplete endogenous centrobin (Figure 10A). I then performed microtubule regrowth assays with the GFP-CBN- and GFP-CBN$^{\Delta C}$-rescued cells. The results showed that intensity and the number of centrosomal microtubules and the number of cytoplasmic microtubules was rescued with GFP-CBN but not with GFP-CBN$^{\Delta C}$ (Figure 10B-E). These results indicate that the C-terminal domain of centrobin is crucial for microtubule nucleation both at the centrosome and cytoplasm.

Microtubule regrowth is limited to the centrosome with the centrobin-PACT fusion protein

In order to limit ectopic centrobin to the centrosome, I linked the PACT (Pericentrin-AKAP450 centrosomal targeting) domain to GFP-CBN (Gillingham and Munro, 2000). As expected, most of GFP-CBN-PACT localized to the centrosome whereas GFP-CBN was detected in both the centrosome and cytoplasm (Figure 11A, B). I adjusted the cellular GFP-CBN and GFP-CBN-PACT levels to comparable to endogenous centrobin. Then, I performed the microtubule regrowth assays with the GFP-CBN- and GFP-CBN-PACT-rescued cells after the depletion of endogenous centrobin (Figure 12A). The results showed that the centrosomal microtubule levels and the number of the centrosomal microtubules were efficiently rescued in both the GFP-CBN- and GFP-CBN-PACT-rescued cells (Figure 12B-D). However, the number of cytoplasmic microtubules was not rescued with GFP-CBN-PACT (Figure 12E). I also performed microtubule regrowth assays with U2OS cells with excess amounts of GFP-CBN-PACT (Figure 13). Excess amounts of GFP-CBN-PACT saturated the centrosomes and even formed small dots in the cytoplasm. Furthermore, microtubules were grown from the cytoplasmic dots as well as from the centrosomes (Figure 13). These results confirm that centrobin is essential for microtubule nucleation irrespective of the subcellular localization.

Importance of NEK2 phosphorylation of centroin in the cytoplasmic microtubule formation

NEK2 phosphorylates centrobin at specific residues including T35, S36, S41 and S45 (Park and Rhee, 2013). The phospho-resistant mutant of centrobin at these residues (CBN^{4A}) enhanced microtubule stabilizing activity, suggesting that NEK2 antagonizes the centrobin activity by phosphorylation (Park and Rhee, 2013). First, I depleted endogenous NEK2 of U2OS cells with siRNA transfection and performed microtubule regrowth assays (Figure 14A, B). The results showed that the microtubule nucleation activities in both the centrosome and cytoplasm were enhanced in the NEK2-depleted cells (Figure 15A-D). Next, I examined whether centrobin mediates the inhibitory effects of NEK2 on microtubule network formation or not. To determine the microtubule nucleating activity of the phospho-resistant centrobin, I generated inducible U2OS cell lines in which the wild type (GFP-CBN) and phospho-resistant mutant (GFP-CBN^{4A}) of centrobin are stably expressed. I, then, depleted the endogenous centrobin and performed microtubule regrowth assays (Figure 16A, B). The results showed that the centrosomal microtubule levels and the number of centrosomal microtubules were enhanced in the GFP-CBN^{4A}-rescued cells in comparison to the GFP-CBN-rescued cells (Figure 16B-D). The number of cytoplasmic microtubules was also enhanced significantly in the GFP-CBN^{4A}-rescued cells (Figure 16E). These results indicate that NEK2 phosphorylation antagonizes the microtubule nucleating activity of centrobin in both the centrosome and cytoplasm.

Centrobin in the mouse hippocampal cells

I observed subcellular localization of centrobin in the mouse hippocampal cells. The centrobin signals were detected at both the mother and daughter centrioles in the young hippocampal cells (2 days in vitro; 2DIV) (Figure 17A, B). This result was unexpected because centrobin is known to localize exclusively at the daughter centrioles in most cells examined (Jeong et al., 2007; Zou et al., 2005). In the mature 12DIV hippocampal cells, additional centrobin signals were also detected out of the centrosomes (Figure 17A, B). However, the additional centrobin dots were not co-immunostained with the γ-tubulin antibody, suggesting that they may not function as microtubule organizing centers (Figure 17C).

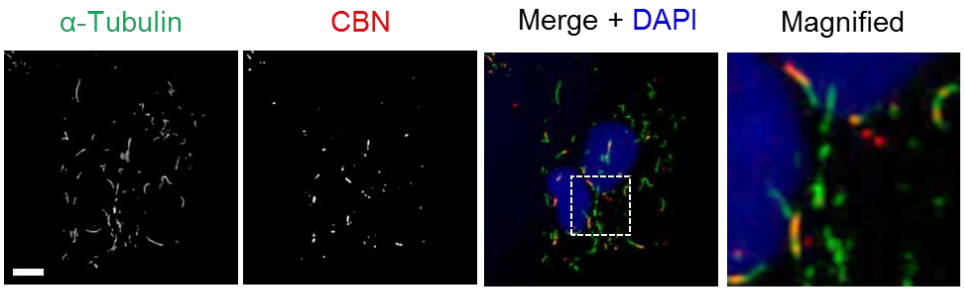


Figure 5. Cytoplasmic distribution of centrobin. U2OS cells were placed on ice for 45 min and coimmunostained with the α-tubulin (green) and centrobin (red) antibodies. Nuclei were stained with DAPI (blue). The magnified view shows the cytoplasmic centrobin along with stable microtubules. Scale bar, 10 μm.

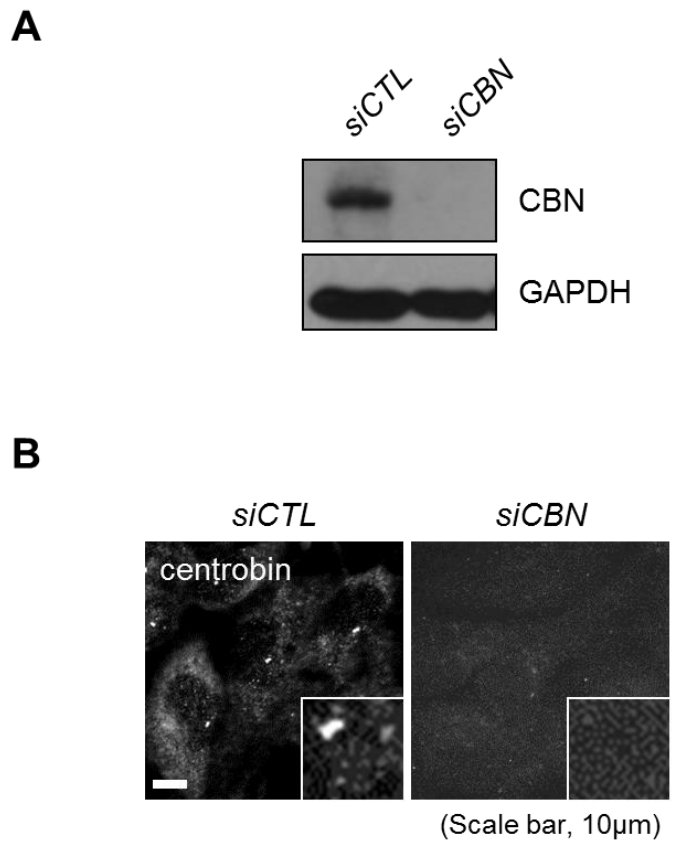


Figure 6. Depletion of endogenous centrobin in U2OS cells. Immunoblot (A) and immunostaining analysis (B) were carried out to confirm centrobin depletion with siRNA transfection. Scale bar, 10 μm. Insets are magnified views of the centrosomes.

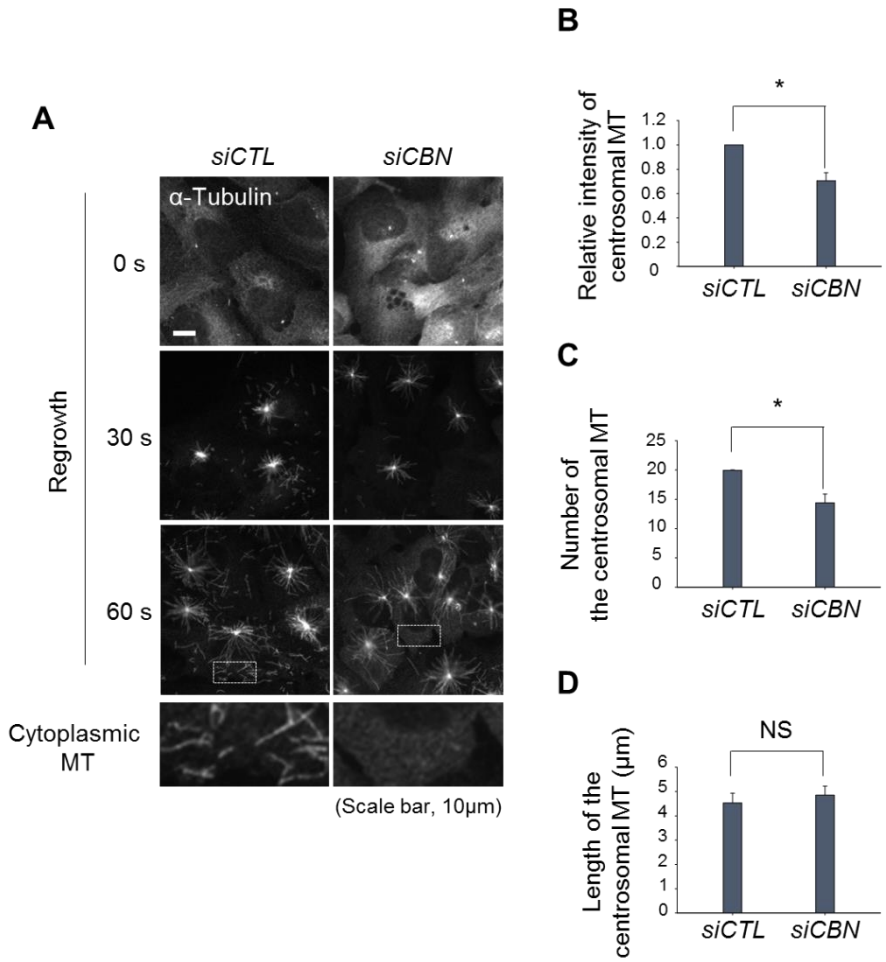


Figure 7. Microtubule regrowth assays in the centrobin-depleted cells. (A) Microtubule regrowth assays were performed with the centrobin-depleted U2OS cells. The cells were immunostained with the α-tubulin antibody. The cytoplasmic microtubules were enlarged from the 60-second time point. The centrosomal microtubule intensities (B), the number (C) and length (D) of microtubules from a centrosome were quantified at the 10-second time point. Scale bar, 10 μm. At least 30 cells per an experimental group were measured in each of three independent experiments. Data show the mean±s.d.. *P<0.05, in comparison to the control.

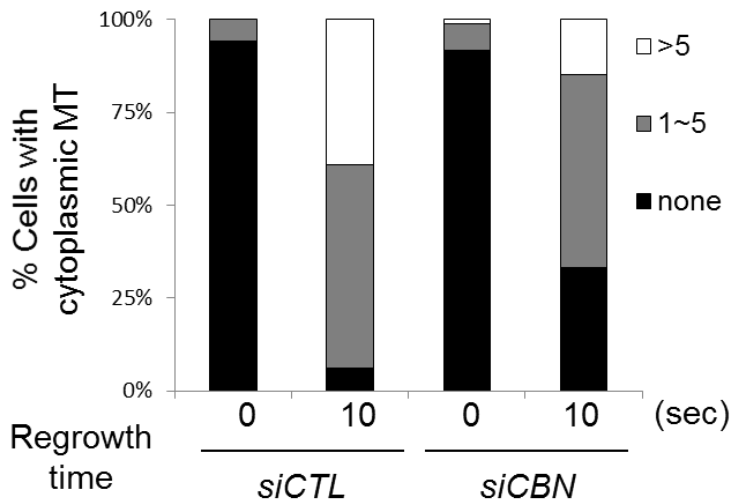


Figure 8. The number of cytoplasmic microtubules in the centrobin-depleted cells.

The number of cells with cytoplasmic microtubules were quantified at the 10-second time point. At least 30 cells per an experimental group were measured in each of three independent experiments.

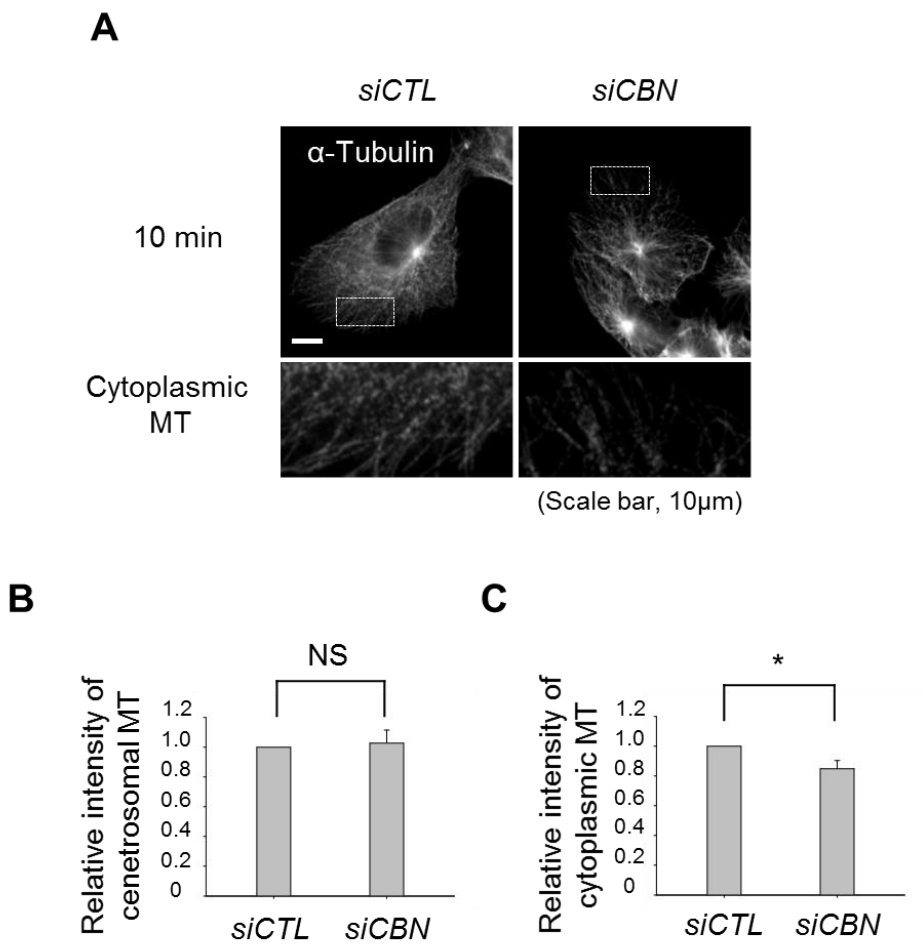


Figure 9. The microtubule regrowth for extended time periods. (A) Microtubule regrowth assays were performed for extended time periods up to 10 min. Microtubule intensities in the centrosome (B) and cytoplasm (C) were determined. Scale bar, 10 μm. At least 30 cells per an experimental group were measured in each of three independent experiments. Data show the mean±s.d.. *P<0.05, in comparison to the control.

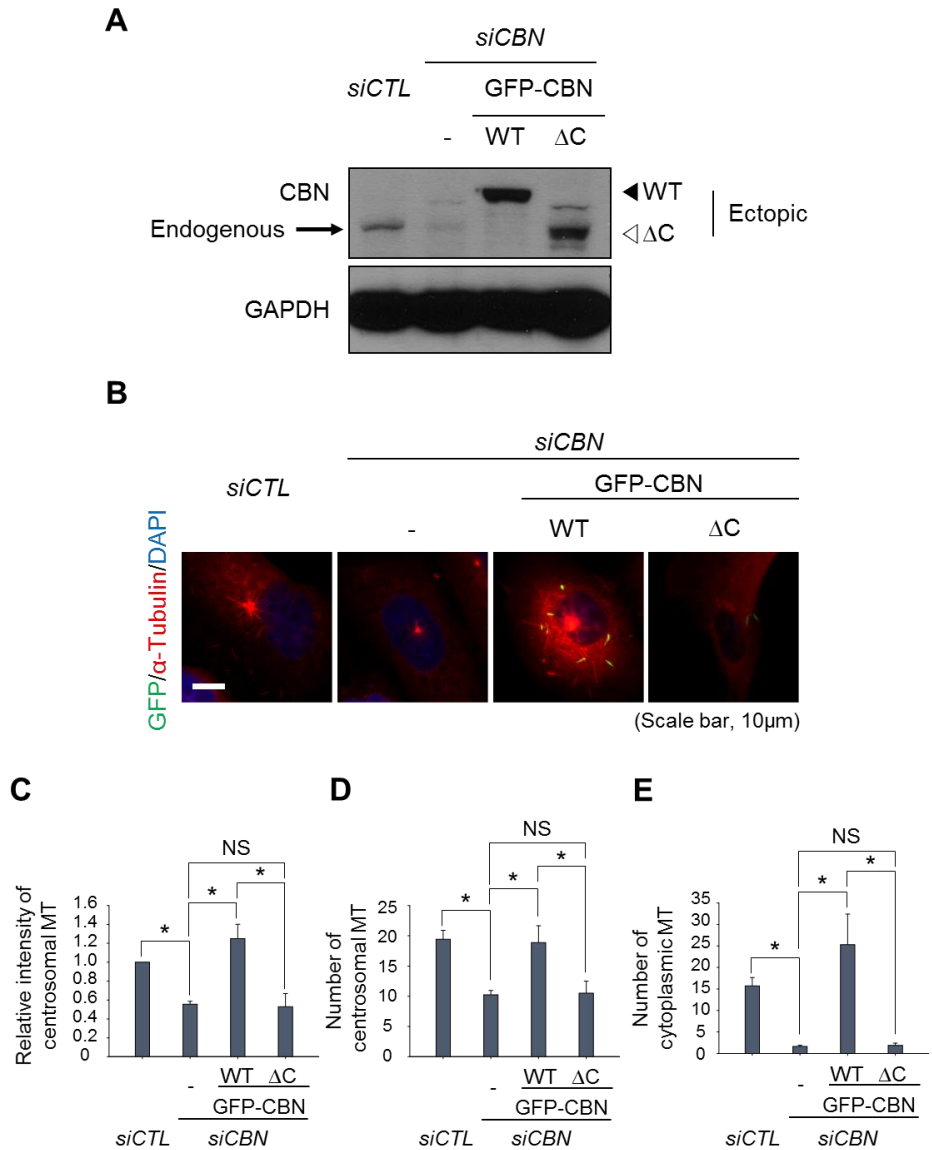


Figure 10. Microtubule regrowth assays with a C-terminus deletion mutant of centrobin. (A) Immunoblot was performed to determine the GFP-CBN and GFP-CBN$^{\Delta C}$ levels in centrobin-depleted U2OS cells. (B) Microtubule regrowth assays were performed with the centrobin-depleted U2OS cells rescued with GFP-CBN or GFP-CBN$^{\Delta C}$. The cells were immunostained with the GFP (green) and α-tubulin (red) antibodies. Scale bar, 10 μm. The centrosomal microtubule intensities (C), the number of microtubules from a centrosome (D), and the number of cytoplasmic microtubules (E) were quantified at the 10-second time point. At least 30 cells per an experimental group were measured in each of three independent experiments. Data show the mean±s.d.. *P<0.05, in comparison to the centrobin-depleted cells.

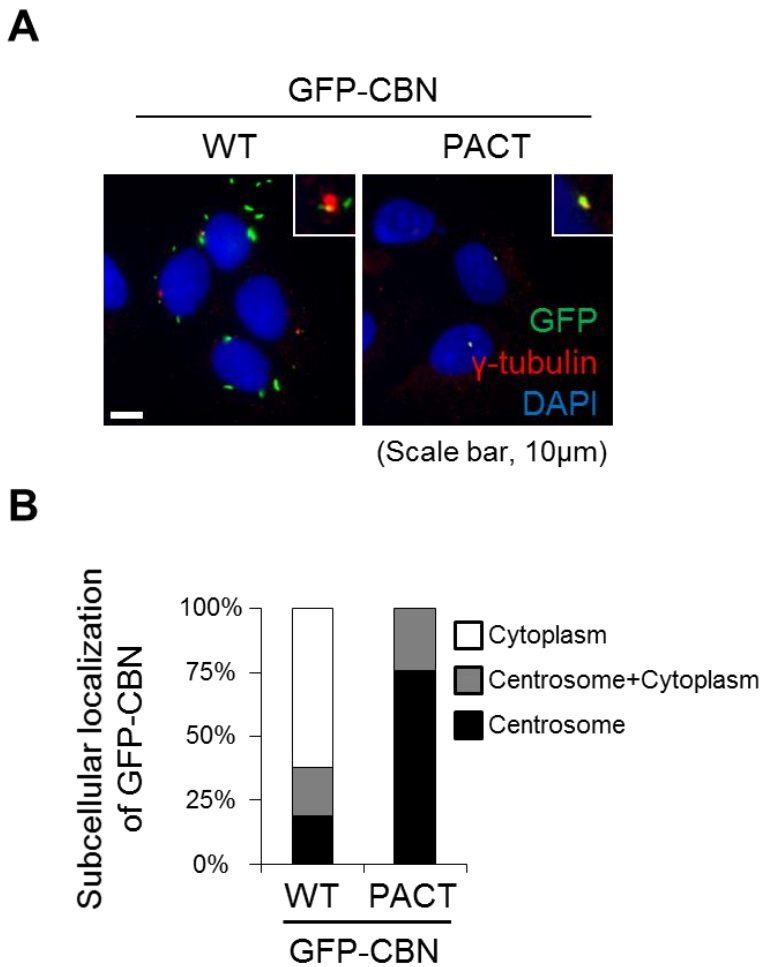


Figure 11. Expression of centrobin-PACT. (A) Immunostaining analysis was performed to determine subcellular distribution of GFP-CBN and GFP-CBN-PACT in U2OS cells. The cells were coimmunostained with the GFP (green) and γ-tubulin (red) antibodies. Nuclei were stained with DAPI (blue). Insets are magnified views of the centrosomes. Scale bar, 10 μm. (B) The number of cells with cytoplasmic and/or centrosomal signals of ectopic GFP-CBN was counted. At least 30 cells per an experimental group were measured in each of three independent experiments.

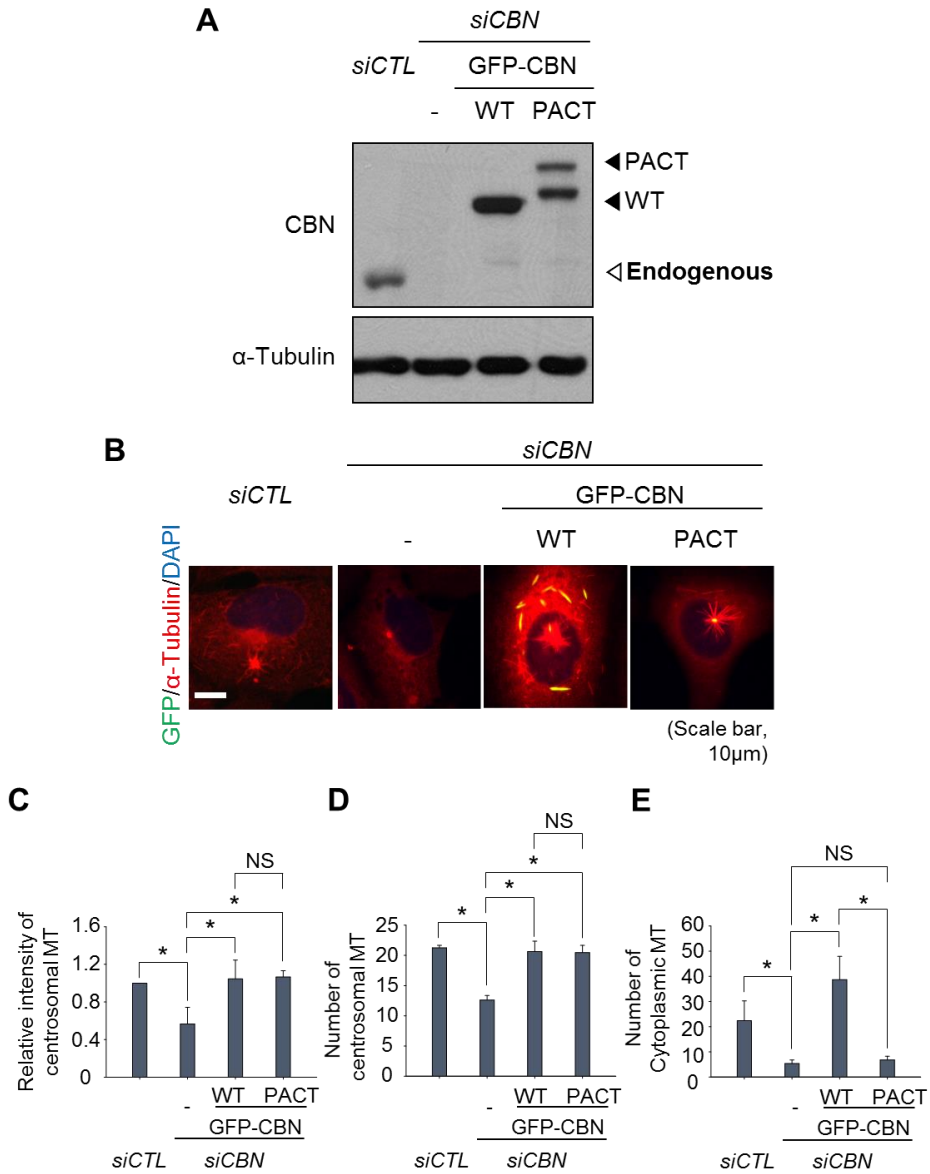


Figure 12. Microtubule regrowth assays with centrobin-PACT. (A) Immunoblot analysis of centrobin was performed with the centrobin-depleted U2OS cells rescued with GFP-CBN and GFP-CBN-PACT. (B) Microtubule regrowth assays were performed with the centrobin-depleted cells rescued with GFP-CBN or GFP-CBN-PACT. The cells were coimmunostained with the GFP (green) and α-tubulin (red) antibodies. Scale bar, 10 μm. The intensity of centrosomal microtubules (C), the number of microtubules from centrosome (D), and the number of microtubules at the cytoplasm (E) were quantified at the 10-second time point. At least 30 cells per an experimental group were measured in each of three independent experiments. Data show the mean±s.d.. *P<0.05, in comparison to the centrobin depleted cells.

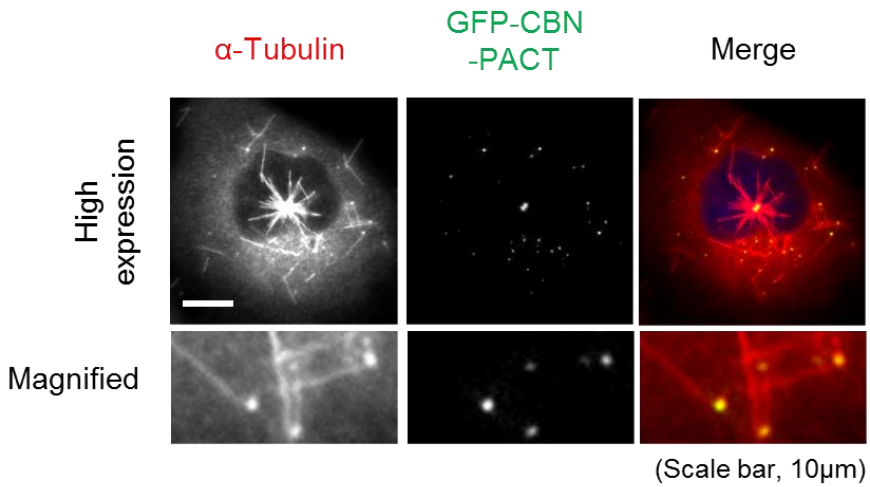


Figure 13. Microtubule regrowth assays with excess amounts of centrobin-PACT. Immunostaining analysis was performed with centrobin-depleted U2OS cells rescued with an excess amount of GFP-CBN-PACT. The magnified view shows the regrown microtubules from the cytoplasmic centrobin. Scale bar, 10 μm.

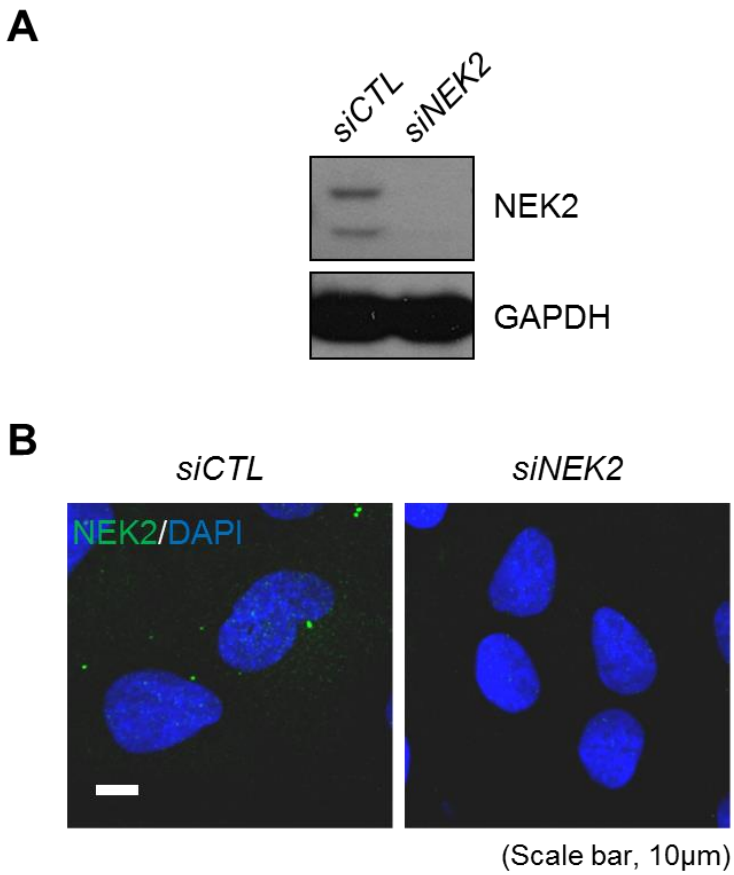


Figure 14. Depletion of endogenous NEK2 in U2OS cells. Immunoblot (A) and immunostaining (B) analysis were carried out to confirm NEK2 depletion with siRNA transfection. Scale bar, 10 μm.

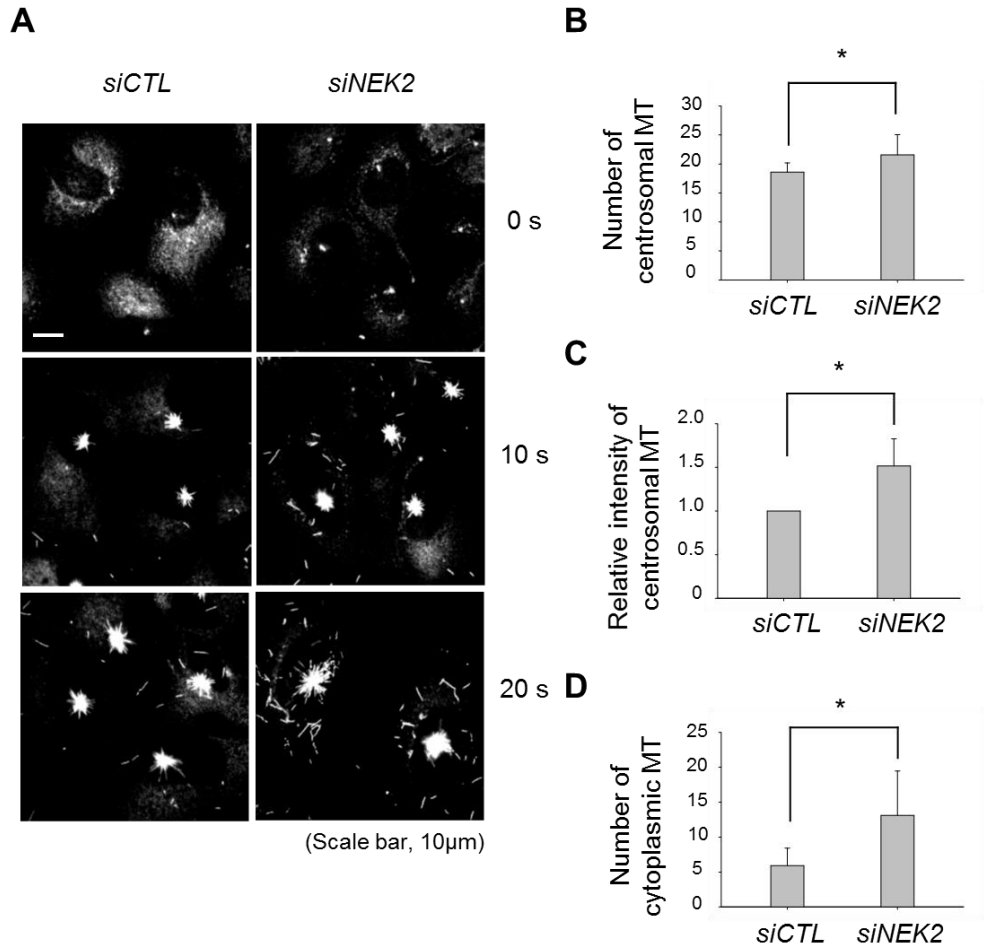


Figure 15. Microtubule regrowth assays in the NEK2-depleted cells. (A) Microtubule regrowth assays were performed with the NEK2-depleted U2OS cells. The cells were immunostained with the α-tubulin antibody. The number of microtubules from a centrosome (B), centrosomal microtubule intensities (C), and the number of cytoplasmic microtubules (D) were quantified. Scale bar, 10 μm. At least 30 cells per an experimental group were measured in each of three independent experiments. Data show the mean±s.d.. *P<0.05, in comparison to the control cells.

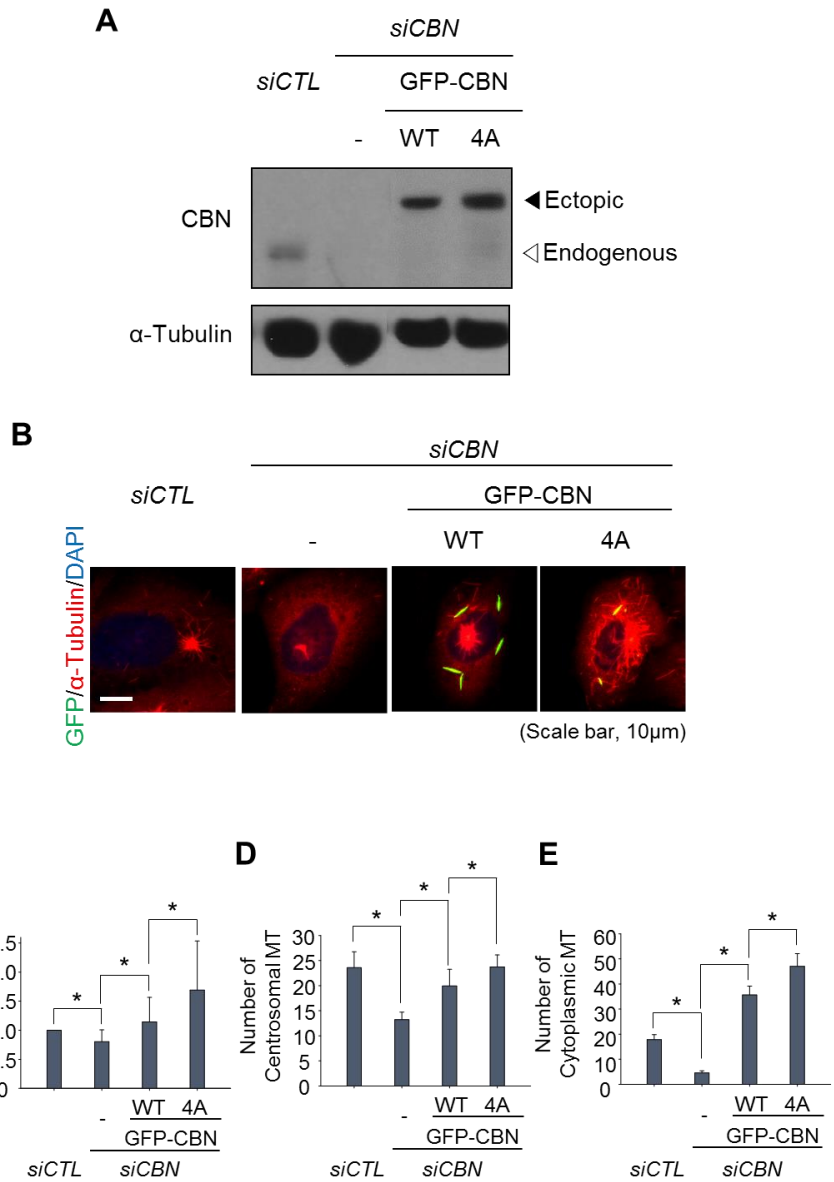


Figure 16. Importance of NEK2 phosphorylation of centrobin in microtubule formation. Centrobin-depleted U2OS cells were stably rescued with the wild type (WT) or a phospho-resistant mutant of centrobin at T35, S36, S41, S45 (4A). (A) Immunoblot analysis was performed with the centrobin and α-tubulin antibodies. (B) Microtubule regrowth assays were performed for 20 seconds with the centrobin-rescued U2OS cells. The cells were coimmunostained with the GFP (green) and α-tubulin (red) antibodies. Scale bar, 10 μm. The centrosomal microtubule intensities (C), number of microtubules from a centrosome (D), and number of cytoplasmic microtubules (E) were quantified at the 10-second time point. At least 30 cells per an experimental group were measured in each of three independent experiments. Data show the mean±s.d.. *P<0.05, in comparison to the centrobin depleted cells.

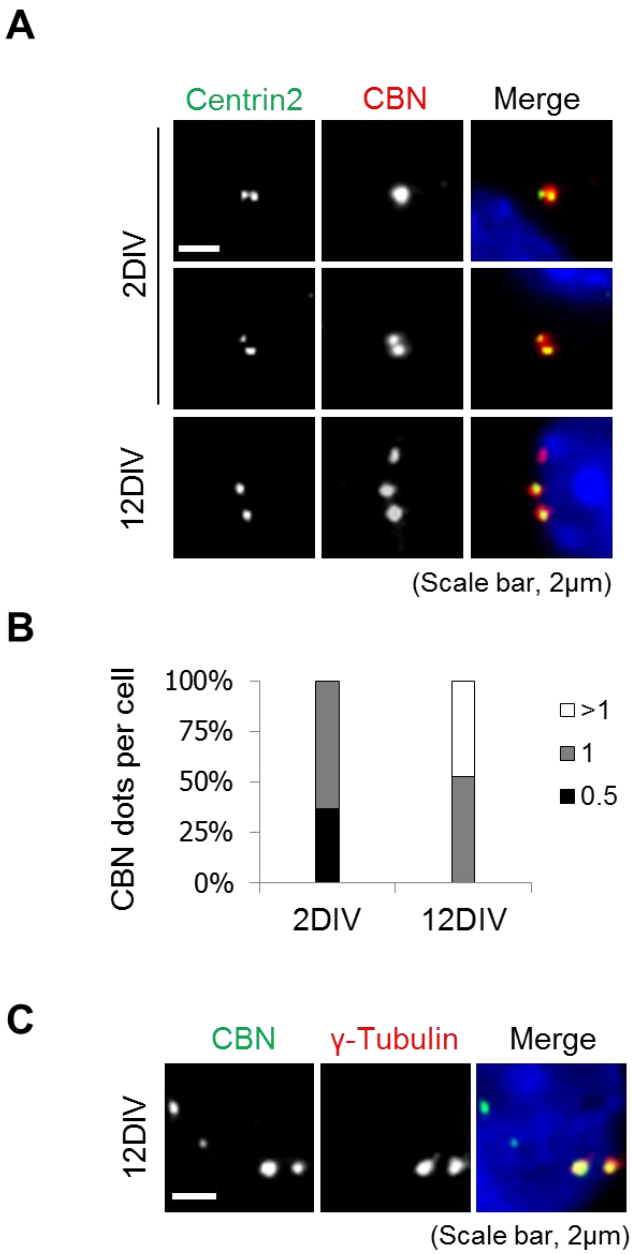


Figure 17. Centrobin expression in mouse hippocampal cells. (A) Mouse hippocampal cells at the 2DIV and 12DIV developmental stages were coimmunostained with the centrobin (red) and centrin2 (green) antibodies. Nuclei were stained with DAPI (blue). Scale bar, 2 μm. (B) The number of centrobin dots in a hippocampal cell was counted. At least 30 cells per an experimental group were measured in each of three independent experiments. Data show the mean±s.d.. (C) The 12DIV mouse hippocampal cells were coimmunostained with the centrobin (green) and γ-tubulin (red) antibodies. Nuclei were stained with DAPI (blue). Scale bar, 2 μm.

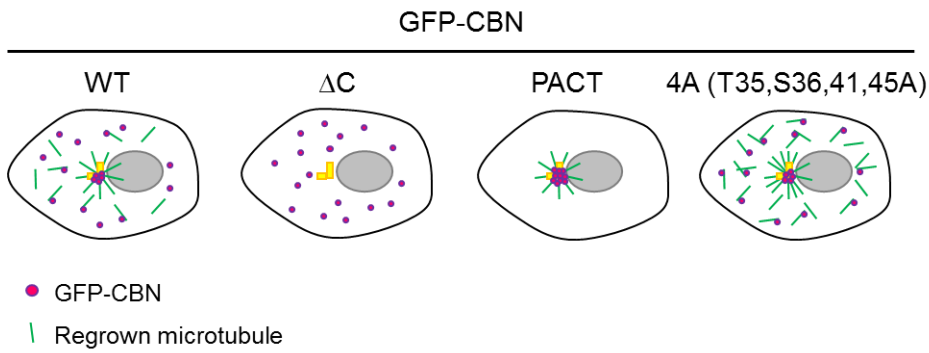


Figure 18. Model. Centrobin in both the centrosome and cytoplasm contributes to microtubule nucleation/stabilization. NEK2 phosphorylation reduces the microtubule nucleation/stabilization activity of centrobin.

Discussion

In this study, I detected a specific localization of cytoplasmic centrobin at the stable microtubules. In hippocampal cells, centrobin forms cytoplasmic dots in addition to the localization at both centrosomes with the mother and daughter centrioles. Such specific localization pattern suggests that cytoplasmic centrobin is not just a reserved pool for centrosomal localization but also has a specific role in the cytoplasm. In fact, centrobin enhanced microtubule formation outside as well as inside the centrosome. These results suggest that cytoplasmic centrobin participates in microtubule network formation in a cell.

Centrobin was initially known to be a daughter centriole-specific protein which is critical for centriole duplication (Jeong et al., 2007; Zou et al., 2005). A known biological activity of centrobin, however, is microtubule nucleation and stabilization (Lee et al., 2010). In fact, I observed that the number of centrosomal microtubules was reduced in centrobin-depleted cells and rescued with the wild type and PACT-linked centrobin. The result indicates that centrosomal centrobin is involved in microtubule nucleation at the centrosome. In consistent with my view, centrobin is essential for the microtubule nucleation in the daughter centrioles of *Drosophila* neuroblasts (Januschke et al., 2013). It is not clear how the microtubule stabilization activity of centrobin is essential for procentriole assembly. It is possible that centrobin might play a role in nucleation and stabilization of the triplet microtubules for procentriole formation. Recently, it was reported that centrobin induces centriole elongation through regulating the CPAP level which is also a crucial factor for centriole growth (Gudi et al., 2015; Gudi et al., 2014).

It remains to be investigated what is the biological significance of cytoplasmic centrobin. It is known that NEK2 and PLK1 phosphorylate centrobin at specific sites and result in opposite outcomes (Lee et al., 2010; Park and Rhee, 2013). NEK2 phosphorylation reduces the microtubule nucleation activity of centrobin while PLK1 phosphorylation enhances it (Lee et al., 2010; Park and Rhee, 2013). In fact, I observed that the phospho-resistant mutant centrobin against NEK2 enhanced the microtubule formation in both centrosome and cytoplasm, implying that centrobin mediates NEK2 regulation on microtubule formation (Figure 18). Since the NEK2 activity is highest at G2 phase, NEK2 may control centrobin functions prior to mitosis, possibly for reorganization of microtubule networks. Once cells enter mitosis, PLK1 becomes active and phosphorylates centrobin. An enhanced centrobin activity may be critical for formation of stable mitotic spindle (Lee et al., 2010). In fact, PLK1 phosphorylation is critical for enhanced microtubule organizing activity of the spindle pole with a daughter centriole during the mitosis of *Drosophila* neuroblasts (Januschke et al., 2013). I currently investigate involvement of centrobin in cellular morphology of non-dividing cells, such as neuronal cells.

Chapter 2.
Involvement of CEP215 in glial differentiation of P19 embryonic carcinoma cells

Abstract

CEP215 is one of the essential PCM proteins which is important for recruitment of γ-TuRC components to the centrosome. CEP215 contributes to the centrosome maturation through its interaction with pericentrrin and γ-tubulin during mitosis, and this progress is essential for bipolar spindle formation. The microtubule nucleation is activated by CEP215 binding to γ-tubulin, and CEP215 is necessary for microtubule nucleation at both the centrosome and cytoplasm. Here I report specific localization of CEP215 along the processes of astrocytes in cultured mouse hippocampal cells and differentiated P19 embryonic carcinoma cells. GFAP expression and process formation were suppressed in *CEP215*-deleted P19 cells. The phenotypes of *CEP215* deletion were rescued by ectopic Flag-CEP215, but not by Flag-CEP215$^{\Delta PCNT}$ and Flag-CEP215^{F75A}, which do not interact with pericentrin and γ-tubulin, respectively. I observed reduction of the centrosomal γ-tubulin levels in *CEP215*-deleted P19 cells. Based on the results, I propose that the microtubule nucleating function of CEP215 is essential for glial differentiation.

Introduction

Centrosome consists of two centrioles and pericentriolar material (PCM) surrounding centrioles. CEP215 is one of the central PCM protein with pericentrin and γ-tubulin (Fong et al., 2008). It was revealed that PCM proteins form toroidal structure by observation using super resolution microscopy (SRM) (Lawo et al., 2012). During mitosis, PCM proteins increase their volume to form bipolar spindle, and this process is called centrosome maturation (Kim and Rhee, 2014; Luders, 2012). CEP215 is essential for centrosome maturation through interaction with other PCM proteins such as pericentrin and γ-tubulin. Therefore, CEP215 depleted cells have a defect in centrosome maturation and fail to form bipolar spindle during mitosis (Kim and Rhee, 2014).

CEP215 is also known as CDK5RAP2 because it was characterized with p35 which is neuron specific activator of CDK5 (Ching et al., 2000). CEP215 is involved in microtubule nucleation through interaction with γ-TuRC (γ-tubulin ring complex) components not only at the centrosome but also in the cytoplasm and *in vitro* (Choi et al., 2010). Actually, microtubule nucleation by γ-TuRC is not activated without CEP215, so CEP215 is also called as γ-TuRC-mediated nucleation activator (γ-TuNA) (Choi et al., 2010). CEP215 is the core protein that enables PCM proteins to recruit γ-TuRC components to microtubule organizing center (MTOC), centrosome.

The function of centrosomal proteins including CEP215 in neuronal cells is comparatively well studied. It is well known that mutation of CEP215 causes microcephaly, and a considerable number of centrosomal proteins such as CPAP are

microcephaly-associated genes (Bond et al., 2005). The defects of CEP215 cause premature differentiation of neuronal progenitor cells, and finally induce decrease of neuronal progenitor cell pool (Buchman et al., 2010). It is caused by failure of normal mitotic spindle pole formation of dividing neuronal progenitor cells. The interaction of CEP215 and pericentrin is also important in this progress (Buchman et al., 2010). Recently, it was also reported that CEP215 regulates morphology of neuronal cells through its microtubule nucleating activity (Yalgin et al., 2015). CEP215 can determine the dendrite branching of *Drosophila* dendritic arborization (da) neurons through its γ-TuRC components recruiting function (Yalgin et al., 2015).

The glial cells protect neurons from injury through interaction with neurons, which is known as neuroprotective function. Importantly, formation of synapse between neurons and astrocytes mediate diverse signaling pathway of nervous system, and finally contribute to physiological function of brain (Zeug et al., 2017). The signaling exchange between neurons and astrocytes can be affected by astrocyte coverage of synapse, which is determined by the morphology of astrocytes (Zeug et al., 2017). Therefore, the structure and morphology of astrocytes are properly regulated by signaling pathway such as Rho GTPase (Zeug et al., 2017). The glial fibrillary acidic protein (GFAP) is astrocyte specific intermediate filament Ⅲ protein (Yang and Wang, 2015), and it is the fundamental cytoskeleton supports astrocyte structure. Increase of GFAP expression is observed in most of neurodegenerative disease, and mutation of GFAP is cause of Alexander disease (Maragakis and Rothstein, 2006).

CEP215 is expressed significantly in most types of glial cells (Issa et al., 2013). Especially, CEP215 is expressed 99% of GFAP expressing astrocytes (Issa et al.,

2013), and according to gene expression data base from isolated astrocytes with high purity, the expression level of CEP215 in astrocyte is more than twice compare to that of neurons (Zhang et al., 2016).

Although some centrosomal proteins are expressed significantly in glial cells, its function has not been studied yet at all. Here, I observed specific cytoplasmic expression of centrosomal protein CEP215 along the processes of astrocytes. The cytoplasmic CEP215 was observed using mouse hippocampal cells and embryonic carcinoma P19 cells which differentiate to neuronal cells by retinoic acid treatment. Especially, CEP215-depleted cells using siRNA and CRISPR-Cas9 system showed defects in not only glial cell differentiation, but also in process formation. The interaction of CEP215 and other PCM proteins such as pericentrin and γ-tubulin was important for CEP215 function in glial differentiation, suggesting that recruiting of γ-TuRC components and microtubule nucleating activity are important for CEP215 function. This is the first study found function of centrosomal proteins in glial differentiation and regulation of astrocyte morphology.

Materials and Methods

Antibodies

The CEP215 and pericentrin antibodies were previously described (Kim and Rhee, 2011; Lee and Rhee, 2010). The antibodies against Tuj1 (MMS-435P-100; Covance), neurofilament (N5139; Sigma) and GFAP (#3670; Cell Signaling) were commercially purchased. The goat γ-tubulin (sc-7396; Santa Cruz) and mouse γ-tubulin (ab11316-100; Abcam) were used for immunocytochemistry and immunoblot, respectively. The antibodies against centrin2, centrobin and GAPDH were purchased from Millipore, Abcam and Ambion, respectively. The Alexa-fluorescence secondary antibodies were purchased from Invitrogen. The mouse and rabbit IgG-HRP antibodies were purchased form Sigma and Millipore, respectively.

Plasmids and RNA interference

The wild type, mutant CEP215 cDNA of Δ1726-1893 (ΔPCNT) and F75A were subcloned into pCMV-3XFLAG vector or pSV40-3XFLAG vector. The siRNA specific to mouse CEP215 (5'-CUCAGUGCAGUGAGGCUAUUATT-3') was purchased from ST Pharm, and was transfected using RNAiMAX (Invitrogen) according to the manufacturer's instruction. Non-specific control siRNA (5'-GCAATCGAAGCTCGGCTAC-3') was used.

Cell culture and Differentiation

The P19 embryonic carcinoma cells were cultured in DMEM (Welgene) supplemented with 10% FBS and antibiotics (ANT-MPT; Invivogen) at 37℃ and 5% CO_2. To induce glial differentiation of P19 cells, 1.0 x 10^5 cells/ml were suspension cultured in bacterial Petri dishes, and treated with 1μM all trans-retinoic acid (R2656; Sigma). After 4 days, the aggregated embryoid bodies were dissociated with 0.05% trypsin-0.53mM EDTA (Welgene), and then replated on the poly-L-lysine (P4707; Sigma) coated dishes with 3.2 x 10^5 cells/ml. The cells were maintained in DMEM supplemented 10% FBS and antibiotics for indicated differentiation stages at 37℃ and 5% CO_2.

Mouse hippocampal cultures were prepared from postnatal day 0-1 (P0-1) mouse pups of either sex as previously described (Beaudoin et al., 2012). Dissociated hippocampi tissues were treated with papain (20 μg/ml) and DNase (10 units/μl) for 20 min at 37 °C. The tissues were then mechanically dissociated by titration with a glass Pasteur pipette. Hippocampal neurons 1 x 10^5cells/ml were plated in MEM (Welgene) supplemented with 0.6 % glucose, 1 mM sodium pyruvate, 1% penicillin-streptomycin (Gibco), 2 mM L-glutamine and 10 % certified-fetal bovine serum (c-FBS) (Gibco) for 4 hours before exchange with Neurobasal media (Gibco) containing 0.5 mM L-glutamine and B27 supplement (Gibco). The cells were maintained in a 5% CO2 incubator at 37 °C for up to 12 days. Every four to seven days, half of the original media was discarded and replaced with fresh Neurobasal media supplemented with 0.5 mM glutamine and B27 supplement.

Generation of knockout cells and stable cell lines

RNA-guided targeting of mouse CEP215 in P19 cells was achieved through transfection of Cas9 vector including guide RNA (gRNA). The Cas9 vector pSpCAS9(BB)-2A-Puro was purchased from Addgene, and gRNA was subcloned as described (Ran et al., 2013). The 23 base pair genomic targeting sequence of mouse CEP215 gRNA was 5'-AAGAGGAAGGAAGGCGCCCTGCC-3'. P19 cells were transfected with 2μg of Cas9 vector containing gRNA using Lipofectamine 3000 (Invitrogen), and selected using 1.5μg/ml of puromycin (Calbiochem) for 2 days, and then puromycin was washed with fresh media. The in-del mutation was detected about 87% of cells using SURVEYOR mutation detection kit (Transgenomic), these cells were seeded by single cell in 96well plate for generation of monoclonal cell line. The used monoclonal *CEP215*-deleted cell was confirmed that the endogenous CEP215 is not detected using both immunoblot and immunocytochemistry. Finally, to determine *CEP215* deletion, the genomic amplicons targeted by Cas9 nuclease were extracted from previous monoclonal cells, and cloned into a plasmid. Two types of deletion were confirmed from all tested amplicons (over 20) using Sanger sequencing.

To generate P19 cell lines stably expressing 3XFLAG-CEP215 constructs, the plasmid DNA was transfected in P19 cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instruction. The P19 cell and P19 CEP215 knockout cell lines stably expressing 3XFLAG-CEP215 constructs were generated using 800μg/ml of G418 (Calbiochem) selection.

Immunoblot analysis

The protein samples for immunoblot were lysed using the cells were lysed using RIPA buffer (150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl at pH 8.0, 10mM NaF, 1mM Na_3VO_4, 1mM EDTA and 1mM EGTA) containing a protease inhibitor cocktail (Sigma-Aldrich, P8340). The samples were subjected to SDS-polyacrylamide gel electrophoresis. The gel was transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 0.1% TBST (Tris-buffered saline TBS with 0.1% Tween 20) for 1-2 h. The membrane was incubated with primary antibody overnight at 4˚C, and then washed with 0.1% TBST 3 times. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature, and then washed with 0.1% TBST 3 times. The ECL solution was treated onto the membrane, and then exposed to an X-ray film.

Immunocytochemistry, image processing and statistical analysis

The cells on the coverslip were fixed with methanol for 10 min and washed with phosphate-buffered saline (PBS) 3 times. The fixed cells were blocked with 3% bovine serum albumin in 0.5% PBST (PBS with 0.5% Triton X-100) for 30 min, incubated with the primary antibody for 1 h, washed with 0.1% PBST 3 times, incubated with the secondary antibody for 30 min, and then washed with 0.1% PBST 3 times. The coverslips were mounted on a slide glass using Prolong gold mounting solution (Invitrogen) after DAPI incubation. The immunostained cells were observed using fluorescence microscope with a CCD (Qi-cam Fast 1394; Qimaging) camera and

processed with ImagePro 5.0 (Media Cybernetics, Inc.) and Adobe Photoshop software. The intensity of centrosomal microtubule was measured by drawing the circle including the nucleated microtubules, and background was excluded by drawing same size circle in the nearest cytoplasm, and statistically analyzed with Student's t-test using SigmaPlot.

Results

Subcellular localization of CEP215 in glial cells

I performed immunocytochemical analysis with young (2DIV) and mature (12DIV) mouse hippocampal cells to determine distribution of CEP215 in glial cells. To distinguish the cell types, I used neurofilament (NF) and GFAP which are specific intermediate filaments for neurons and astrocytes, respectively. The results showed that CEP215 was detected at the centrosomes of all cultured hippocampal cells (Figure 19). Furthermore, specific CEP215 signals were detected at the processes of astrocytes which were coimmunostained with GFAP (Figure 19B). On the contrary, no CEP215 signal was detected at the axons and dendrites of neurons which were coimmunostained with NF (Figure 19A). These results indicate that CEP215 is specifically expressed along the processes of astrocytes.

To further investigate the astrocyte-specific CEP215 expression, I used P19 cell, which is a mouse embryonic carcinoma cell. Retinoic acid induces differentiation of P19 cells into both neuronal and glial cells at specific time frames (Lyu et al., 2003). Neurogenesis initiates as soon as the RA-treated cells start to culture in poly-L-lysine coated dishes, whereas gliogenensis takes 6 more days after the culture (Figure 20A). As results, GFAP started to express late at about the time when NF expression was gradually reduced (Figure 20B).

I observed total protein levels of CEP215 during glial differentiation of P19 cells. The immunoblot analysis revealed that the total protein levels of CEP215 decreased during glial differentiation of P19 cells (Figure 21A). It is known that the

centrosomal levels of selected PCM proteins, including CEP215, decrease during neurogenesis (Stiess et al., 2010). To determine centrosomal CEP215 expression during glial differentiation of P19 cells, I performed immunocytochemistry of CEP215 (Figure 21B). The quantified data showed that the intensity of centrosomal CEP215 decreased by a half after glial differentiation, in comparison to undifferentiated cells (Figure 21C). However, the cytoplasmic CEP215 was continuously detected along the processes of GFAP expressing cells even until D17, very late phase of differentiation (Figure 22). The cytoplasmic CEP215 was not detected in the Tuj1-positive neurons. Taken together, these results suggest that, while the centrosomal CEP215 decreased, the cytoplasmic CEP215 is significantly maintained during gliogenesis.

To determine the specificity of the cytoplasmic CEP215, I performed immunocytochemistry with other PCM proteins such as pericentrin and γ-tubulin in P19 cells. Interestingly, both pericentrin and γ-tubulin were detected along the processes of astrocytes similar to CEP215 (Figure 23A). However, the centriole-specific centrin2 was not detected on the processes of astrocytes. These results imply that only PCM proteins function on the processes during gliogenesis. In mature 12DIV hippocampal cells, the cytoplasmic pericentrin and γ-tubulin were clearly detected along the processes (Figure 23B), but they were not specific to astrocyte but also detected at neurons, too. Careful examination of staining pattern suggests that CEP215 did not overlap with pericentrin nor γ-tubulin in the processes (Figure 23B). These results suggest that PCM proteins such as CEP215, pericentrin and γ-tubulin are significantly detected along the processes of astrocyte during gliogenesis, but only CEP215 is astrocyte-specific.

Defects of glial differentiation in CEP215-depleted cells

To investigate importance of CEP215 during glial differentiation, I depleted CEP215 using a mouse CEP215-specific siRNA (*siCEP215*). To deplete CEP215 throughout the period of differentiation, I transfected siRNA before differentiation, and just before gliogenesis in P19 cells (Figure 24A). Immunoblot analysis was performed at D12 differentiation stage to confirm the depletion of CEP215 (Figure 24B). Interestingly, the expression of GFAP was also reduced in CEP215-depleted cells, suggesting suppression of glial cell differentiation.

To further analyze the defects of glial differentiation in CEP215-depleted cells, I performed immunocytochemistry of GFAP. The result showed that the number of GFAP-expressing cells was significantly reduced in CEP215-depleted cells (Figure 25A). While most of the GFAP-positive cells organized processes in control cells at D12, CEP215-depleted cells failed to form processes (Figure 25A). The quantified data indicated that about 20% of the control P19 cells were GFAP-positive at D12, whereas only 10% of the CEP215-depleted cells were GFAP-positive (Figure 25B). Furthermore, CEP215-depleted cells hardly formed processes even if they expressed GFAP (Figure 25B). These results imply that CEP215 is essential not only for GFAP expression but also for process formation during glial differentiation.

Inhibition of glial differentiation in *CEP215* knockout cells

To investigate importance of CEP215 throughout glial differentiation, I generated *CEP215* knockout P19 cells using CRISPR-Cas9 system. The CRISPR-Cas9 system is convenient gene engineering tool that deletion of specific gene is possible by

just simple transfection of guide RNA (gRNA) (Ran et al., 2013). The gRNA I used for generation of *CEP215*-deleted P19 cell targets around 10th amino acids of mouse CEP215 (Figure 26A). I confirmed two types of deletion were established in all the genomic DNA sequence I investigated from monoclonal cell line (Figure 26B). I confirmed that endogenous CEP215 is not detected at all in undifferentiated *CEP215*-deleted P19 cells using both immunoblot (Figure 27A) and immunocytochemistry (Figure 27B).

Next, I induced glial differentiation of the *CEP215*-deleted P19 cells. Like CEP215-depleted cells using siRNA, GFAP expression was significantly reduced in *CEP215*-deleted P19 cells (Figure 28). The immunocytochemistry was also performed to analyze the morphology of astrocytes in *CEP215*-deleted cells in D12. The results showed that the number of GFAP-expressing cells decreased to 5% in *CEP215*-deleted cells in D12 (Figure 29B). At the same time, processes were hardly formed in the *CEP215*-deleted cells (Figure 29). I performed a time-course experiment to determine whether glial differentiation is inhibited or delayed in *CEP215*-deleted P19 cells. The results showed that the suppressed GFAP-positive astrocytes in *CEP215*-deleted cells were not recovered throughout long period of differentiation (Figure 30). These results suggest that glial differentiation is completely inhibited in *CEP215*-deleted cells.

Importance of CEP215 interaction with PCM proteins in gliogenesis

CEP215 is known to interact with a number of proteins for its functions in the centrosome and cytoplasm. Especially, the importance of CEP215 interaction with pericentrin and γ-tubulin for mitotic spindle pole formation is well defined in dividing

cells (Kim and Rhee, 2014). In order to examine whether CEP215 interactions with the PCM proteins are also important for glial differentiation, I generated CEP215 mutants cannot interact with pericentrin and γ-tubulin. They are Flag-CEP215$^{\Delta PCNT}$ whose C-terminal 1726-1893 amino acids are deleted and Flag-CEP215^{F75A} cannot interact with pericentrin and γ-tubulin, respectively (Choi et al., 2010; Kim and Rhee, 2014). Then, I generated P19 cells stably expressing ectopic Flag-CEP215 (WT), Flag-CEP215$^{\Delta PCNT}$ and Flag-CEP215^{F75A}. Then, endogenous CEP215 was depleted using three times of siRNA transfection during P19 cell glial differentiation (Figure 31A). The immunoblot analysis revealed depletion of endogenous CEP215 and expression of ectopic Flag-CEP215 constructs at D12 and D15 differentiation stages (Figure 31B). In this condition, the GFAP expression in CEP215-depleted cells was rescued by ectopic CEP215 (Figure 31B). On the other hand, Flag-CEP215$^{\Delta PCNT}$ and Flag-CEP215^{F75A} mutants did not rescue GFAP expression (Figure 31B). NF expression was not affected irrespective of the presence or absence of CEP215, suggesting that CEP215 has no effect on neurogenesis of P19 cells (Figure 31B).

I also generated *CEP215*-deleted cell lines stably expressing Flag-CEP215, Flag-CEP215$^{\Delta PCNT}$ and Flag-CEP215^{F75A} to study function of CEP215 mutant constructs in *CEP215*-deleted condition. The immunocytochemistry performed in undifferentiated cells showed that the expression level of ectopic proteins was comparable to endogenous CEP215 (Figure 32). As reported, Flag-CEP215$^{\Delta PCNT}$ did not localize at the centrosome (Kim and Rhee, 2014). The immunoblot result showed that GFAP expression was rescued in Flag-CEP215-expressing cells, but not in Flag-CEP215$^{\Delta PCNT}$- and Flag-CEP215^{F75A}-rescued cells at D12 differentiation stage (Figure

33). Although the expression of GFAP was considerably recovered in Flag-CEP215$^{\Delta PCNT}$ -and Flag-CEP215^{F75A} -rescued cells at D15 differentiation stage, it was still low in comparison to Flag-CEP215-rescued cells (Figure 33 short exposure). Taken together, these results suggest that CEP215 interactions with pericentrin and γ-tubulin are essential for its function for glial differentiation.

I determined glial differentiation and cellular morphology using immunocytochemistry in Flag-CEP215-, Flag-CEP215$^{\Delta PCNT}$- and Flag-CEP215^{F75A} -rescued cells. The decreased GFAP-positive cells were rescued by CEP215 WT, but not in Flag-CEP215$^{\Delta PCNT}$ -and Flag-CEP215^{F75A} -rescued cells at both D12 and D15 differentiation stages (Figure 34A). Surprisingly, even process formation was rescued by ectopic Flag-CEP215, but not by Flag-CEP215$^{\Delta PCNT}$ and Flag-CEP215^{F75A} (Figure 34B). These results indicate that interaction of CEP215 with pericentrin and γ-tubulin is essential not only for glial differentiation but also for process formation of astrocytes.

Importance of centrosomal CEP215 and γ-tubulin in gliogenesis

To study MTOC activity of centrosome during gliogenesis, I examined expression of centrosomal pericentrin and γ-tubulin during P19 cell gliogenesis. I firstly investigated total protein level of pericentrin and γ-tubulin using immunoblot assay. While cellular pericentrin level decreased, γ-tubulin was maintained throughout gliogenesis of P19 cell (Figure 35A). The immunocytochemistry assay revealed that centrosomal pericentrin significantly decreased after glial differentiation (Figure 35B, C). However, the centrosomal γ-tubulin expression was maintained before and after differentiation (Figure 35D). It was unexpected because centrosomal γ-tubulin reduces

in other types of differentiated cells such as neurons and keratinocytes (Muroyama et al., 2016; Stiess et al., 2010). As a result, microtubule nucleation activity at the centrosome reduces in differentiated cells. Therefore, this result implies that microtubule nucleating function of centrosome is necessary for astrocyte contrary to other types of differentiated cells.

Next, the centrosomal PCM level was investigated in *CEP215*-deleted cells. While the centrosomal pericentrin was not affected by *CEP215* deletion (Figure 35B, C), the centrosomal γ-tubulin significantly decreased in *CEP215*-deleted cells irrespective of differentiation state (Figure 35B, D). These results suggest that CEP215 is necessary for centrosomal γ-tubulin which is maintained during gliogenesis.

For more clear understanding, the centrosomal pericentrin and γ-tubulin were investigated in Flag-CEP215-, Flag-CEP215$^{\Delta PCNT}$- and Flag-CEP215^{F75A}-rescued cells. The centrosomal pericentrin was not affected by the presence or absence of CEP215 irrespective of differentiation state (Figure 36A). Contrary to pericentrin, reduced centrosomal γ-tubulin was rescued by CEP215 WT, but it was not rescued in Flag-CEP215$^{\Delta PCNT}$ -and Flag-CEP215^{F75A}-expressing cells (Figure 36B). The centrosomal γ-tubulin may not be recovered in Flag-CEP215$^{\Delta PCNT}$-rescued cells, because Flag-CEP215$^{\Delta PCNT}$ cannot localize at the centrosome. Therefore, it can be concluded that centrosomal γ-tubulin is essential for glial differentiation and process formation of astrocytes. Based on these results, it can be assumed that microtubule nucleation depending on CEP215 is essential for glial differentiation.

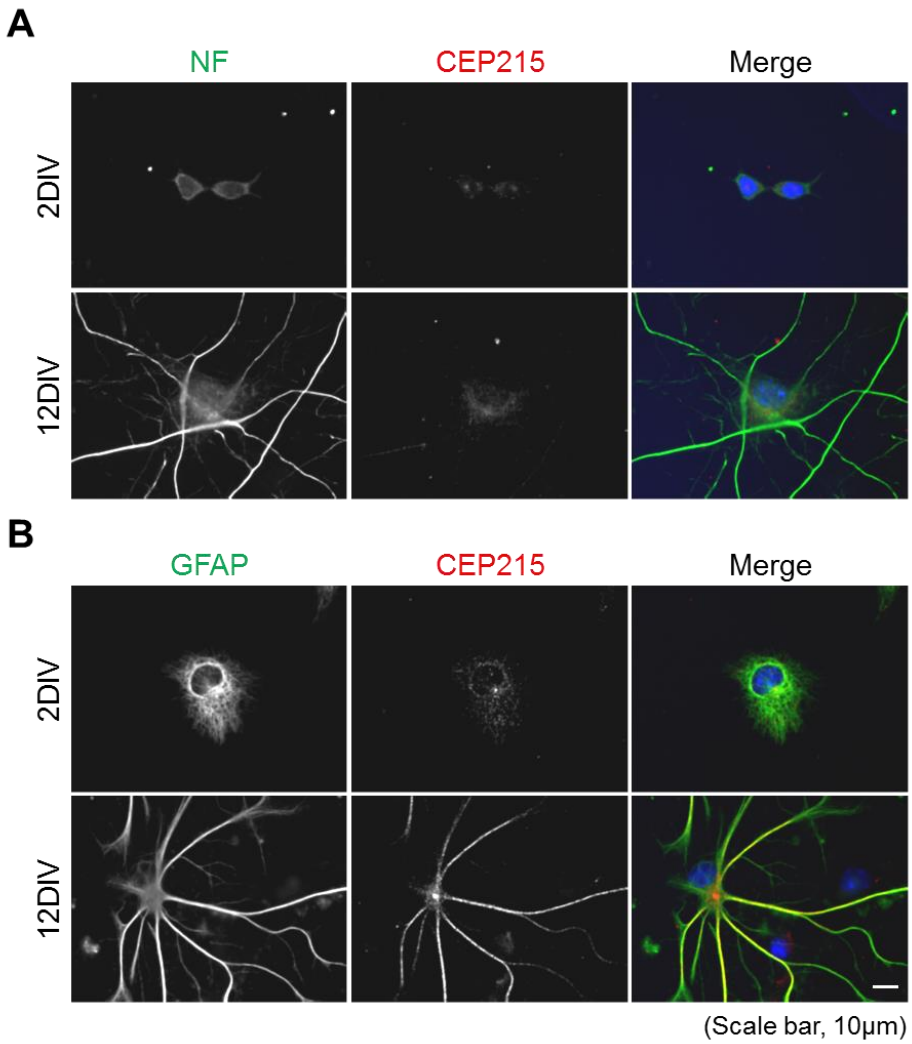


Figure 19. Subcellular localization of CEP215 in mouse hippocampal cells. Cultured mouse hippocampal cells at early (2DIV) and late (12DIV) developmental stages were immunostained with CEP215 (red) antibody, along with the (A) NF or (B) GFAP (green) antibodies. Nuclei were stained with DAPI (blue). Scale bar, 10 μm.

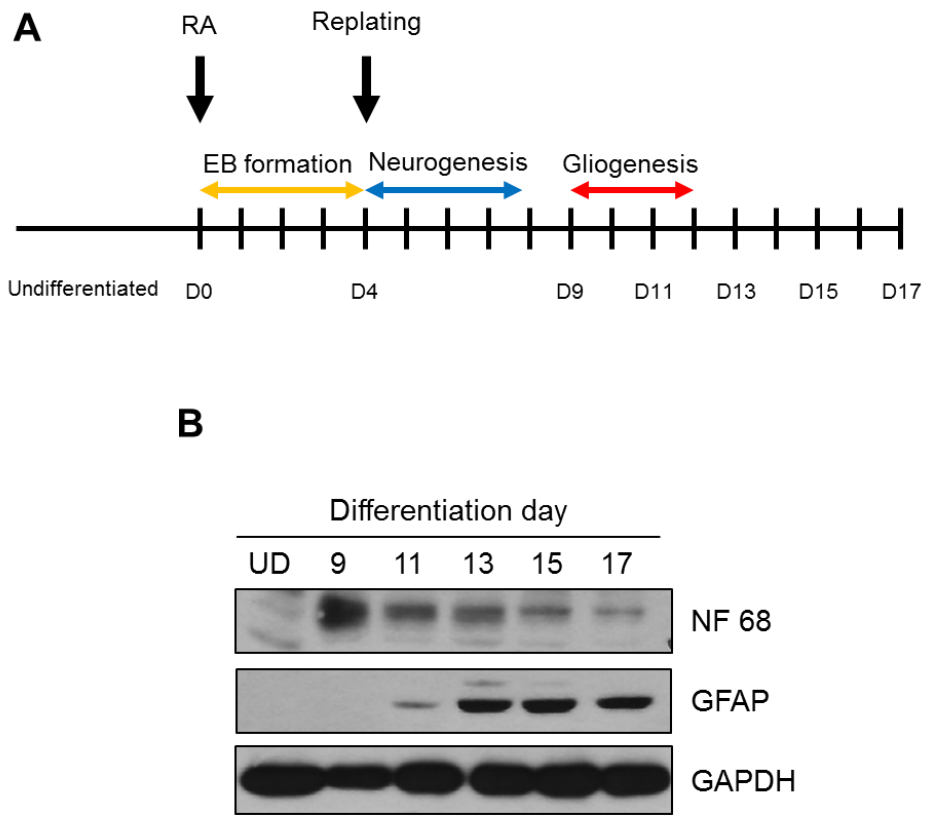


Figure 20. RA-induced differentiation of P19 cells. (A) Experimental scheme of RA-induced differentiation of P19 cells. The cells were treated with RA and induced embryoid bodies in bacterial culture dishes for 4 days (D4). The cells were re-plated on poly-L-lysine-coated dishes for initiation of differentiation. Neurogenesis immediately initiates after the re-plating and gliogenesis initiates in 6 days later (D9). (B) Immunoblot analysis of P19 cells undergoing differentiation. Cell lysates were prepared at indicated days, and subjected to immunoblot analysis with antibodies specific to NF, GFAP and GAPDH.

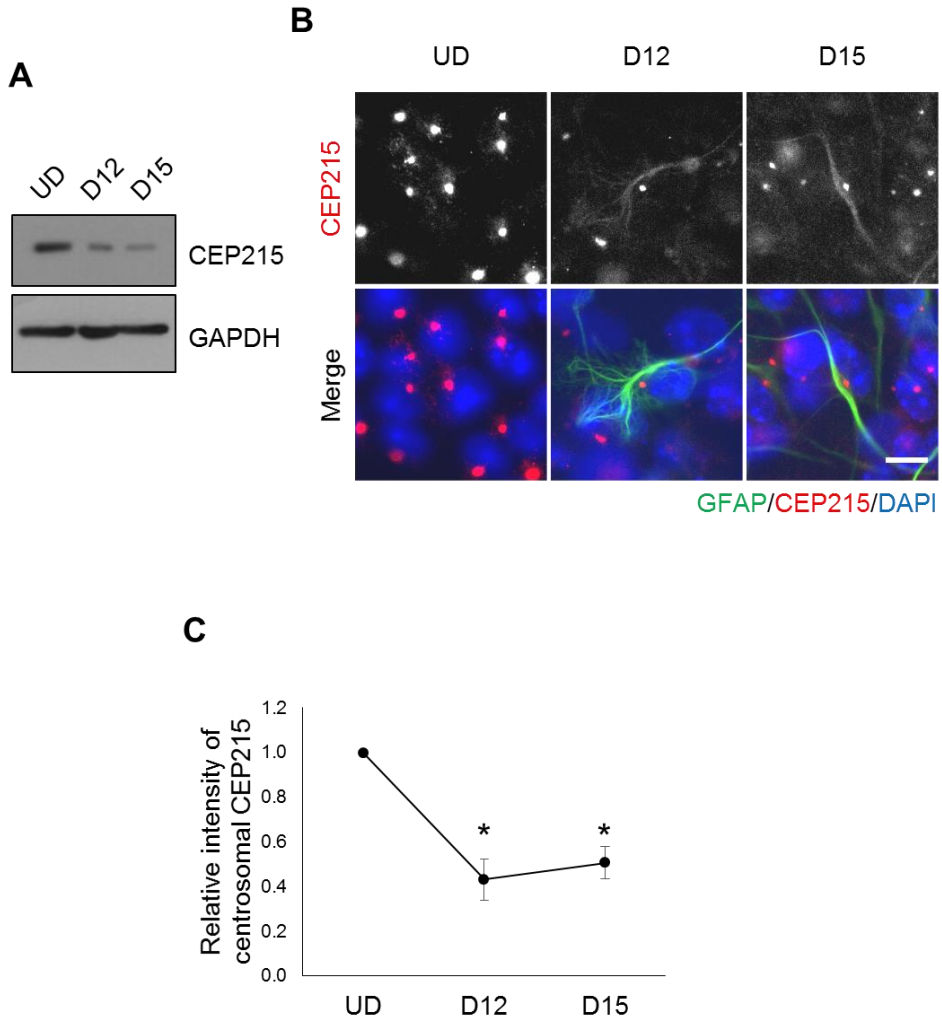


Figure 21. Subcellular localization of CEP215 during glial differentiation of P19 cells. (A) Immunoblot was performed to determine the cellular CEP215 levels in undifferentiated (UD) and differentiated (D12 and D15) P19 cells. (B) P19 cells undergoing differentiation were immunostained with antibodies specific to CEP215 (red) and GFAP (green). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (C) Intensities of the centrosomal CEP215 signals were quantified. More than 90 cells per experimental group were measured in three independent experiments. Error bars; SEM. *P<0.05.

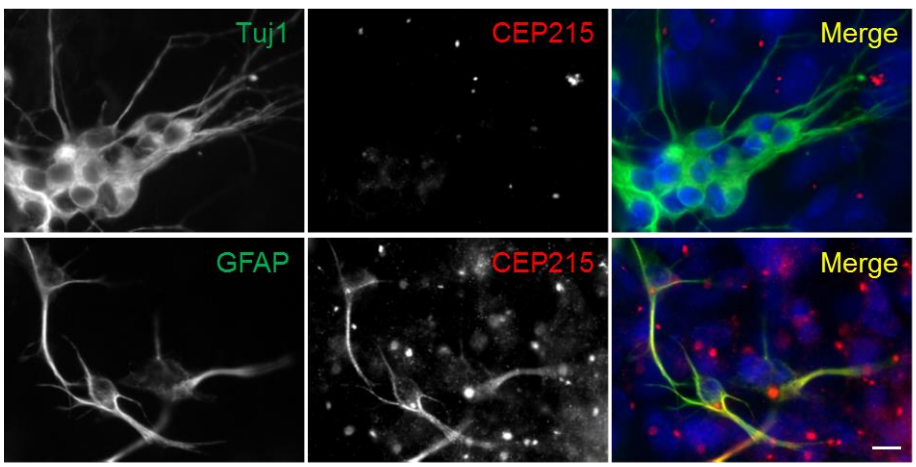


Figure 22. Subcellular localization of CEP215 in differentiated P19 cells. P19 cells at D17 were immunostained with the CEP215 antibody (red) along with the Tuj1 or GFAP antibodies (green). Nuclei were stained with DAPI (blue). Scale bar, 10 μm.

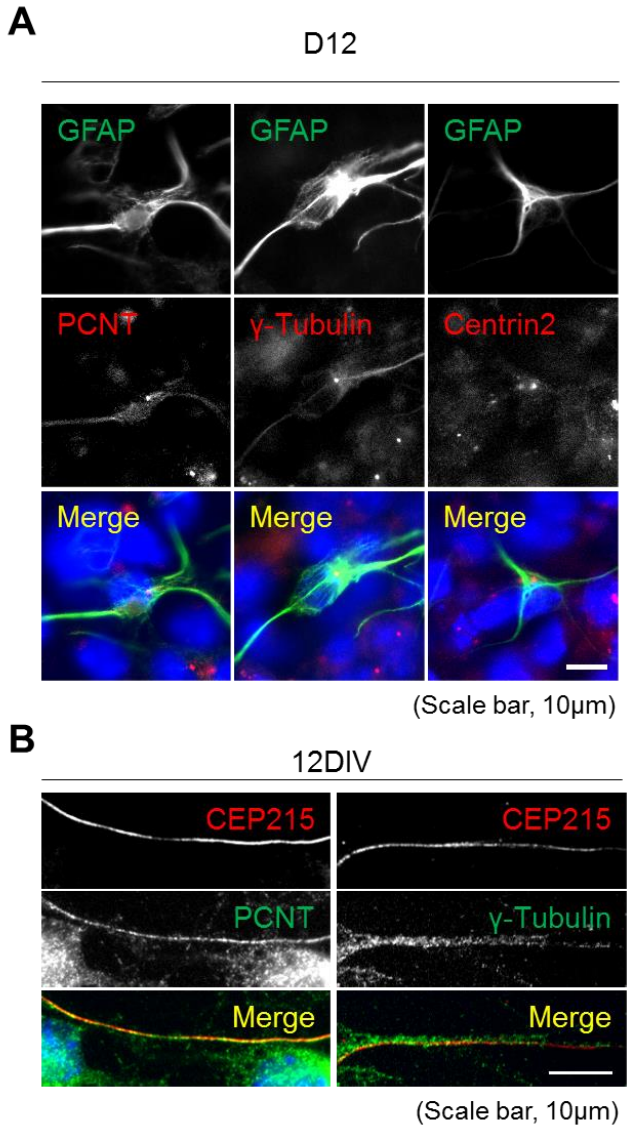


Figure 23. Localization of the centrosomal proteins in the processes of glial cells. (A) P19 cells undergoing differentiation were immunostained with the GFAP antibody (green) along with the pericentrin (PCNT), γ-tubulin or centrin2 antibodies (red). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) Cultured mouse hippocampal cells at 12DIV were coimmunostained with the CEP215 (red) antibody along with the pericentrin and γ-tubulin (green) antibodies. Scale bar, 10 μm.

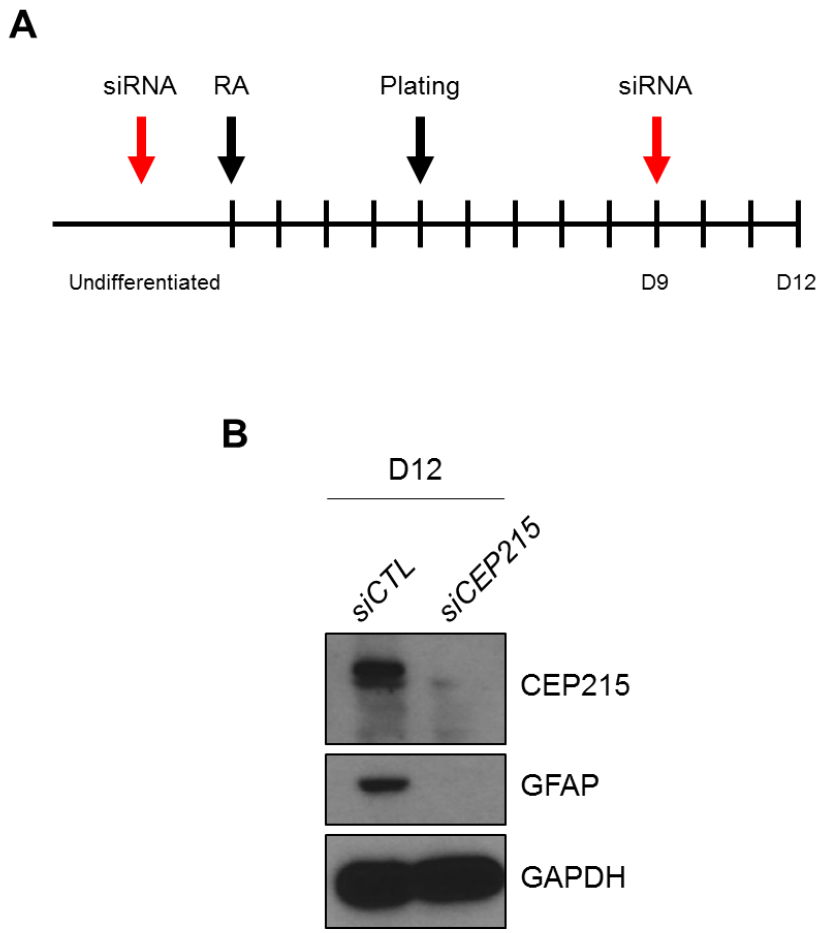


Figure 24. GFAP expression in the CEP215-depleted cells. (A) Experimental scheme. To deplete CEP215 in P19 cells, specific siRNA (*siCEP215*) was transfected twice before (UD) and after (D9) differentiation. (B) Cell lysate at D12 were subjected to immunoblot analysis with antibodies specific to CEP215, GFAP and GAPDH.

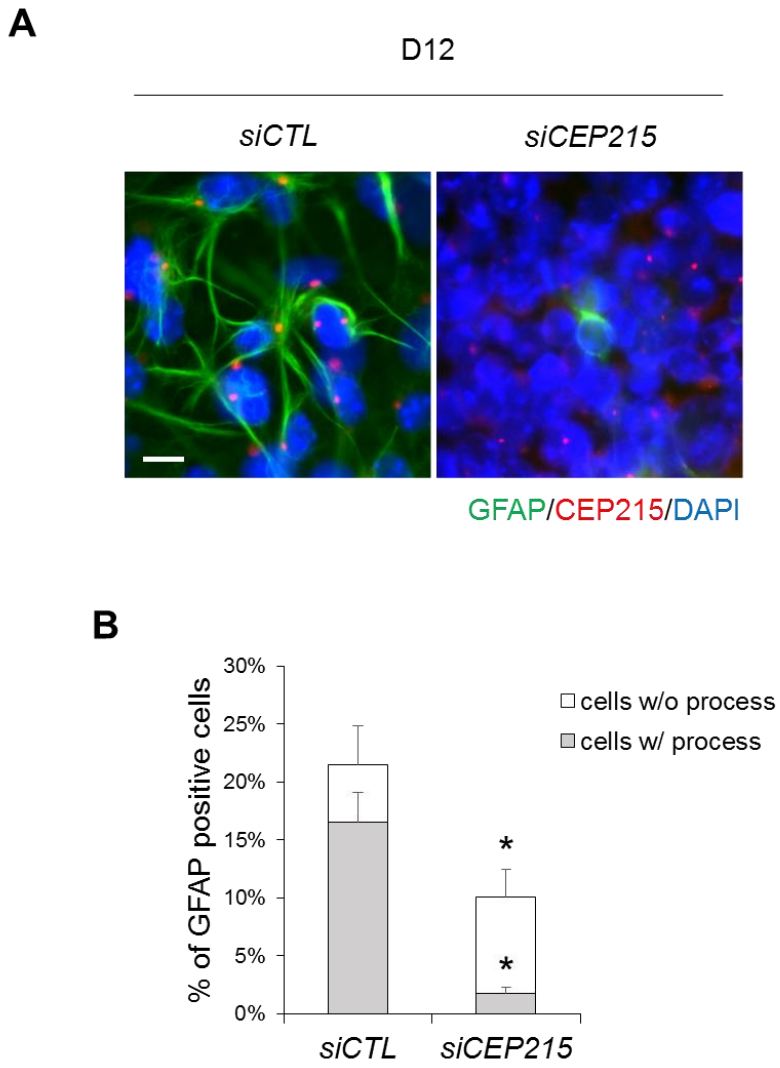
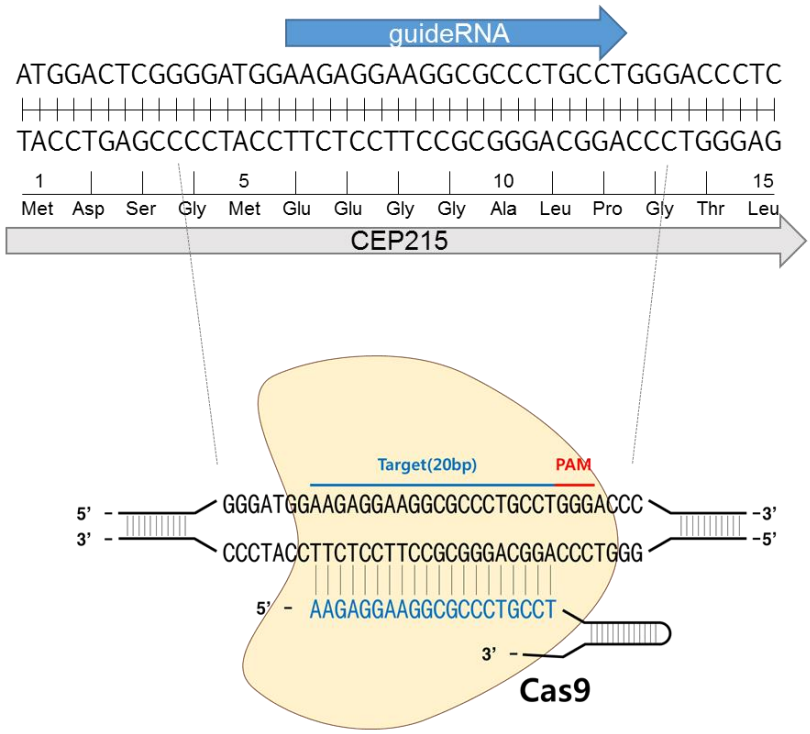


Figure 25. Glial differentiation of the CEP215-depleted P19 cells. (A) CEP215-depleted P19 cells at D12 were coimmunostained with the GFAP (green) and CEP215 (red) antibodies. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) The number of GFAP-positive cells with and without processes were counted. At least 400 cells per experimental group were counted in three independent experiments. Error bars; SEM. *P<0.05.

A



B

Indel types	Sequence	
No indel	GGCGCCCTGCCTGGGACCCTC	0/20(0%)
1bp deletion	GGCGCCCTGC - TGGGACCCTC	4/20(20%)
4bp deletion	GGCGCCCTG - - - - GGACCCTC	16/20(80%)

Figure 26. Generation of *CEP215* knockout P19 cells using the CRISPR-Cas9 system. (A) The guide RNA targeted at 6-13 residues of *CEP215*. (B) In-del types detected at near the gRNA-targeted sites in the *CEP215*-deleted P19 cells.

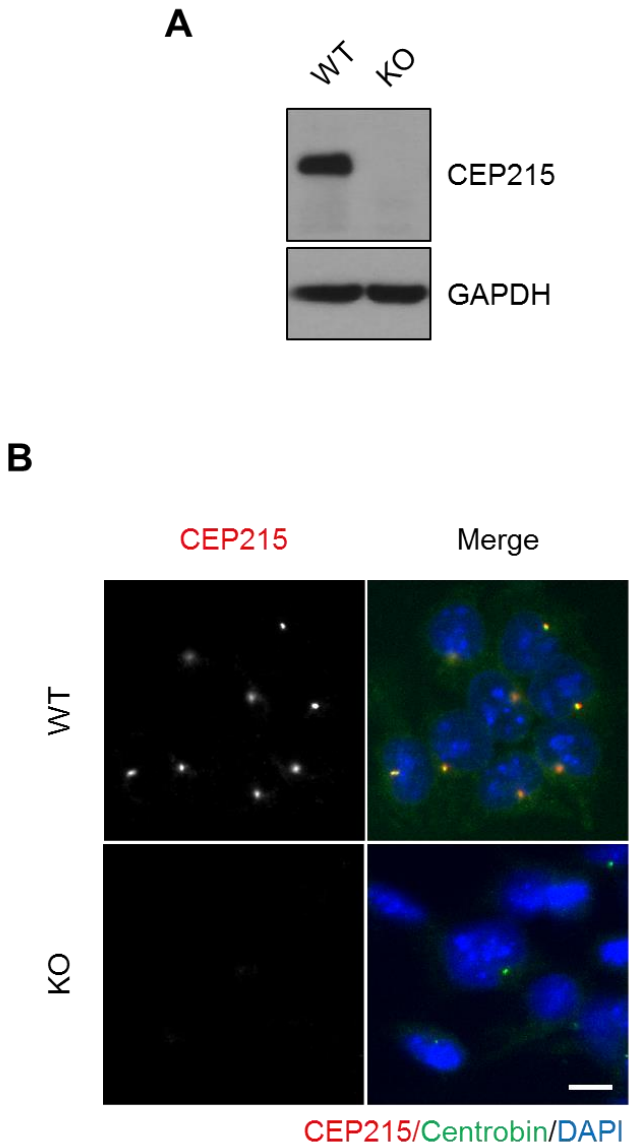


Figure 27. Confirmation of *CEP215* deletion in P19 cells. (A) Immunoblot and (B) immunostaining analyses were carried out to confirm no CEP215 protein expression in the *CEP215*-deleted P19 cell line. Scale bar, 10 μm.

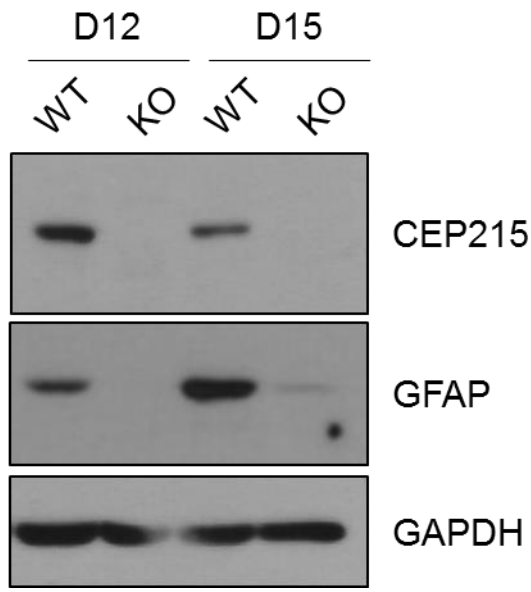


Figure 28. GFAP expression in the *CEP215*-deleted P19 cells. Immunoblot analysis was performed to determine CEP215 and GFAP expression in the *CEP215*-deleted P19 cells at D12 and D15. GAPDH was determined as a control.

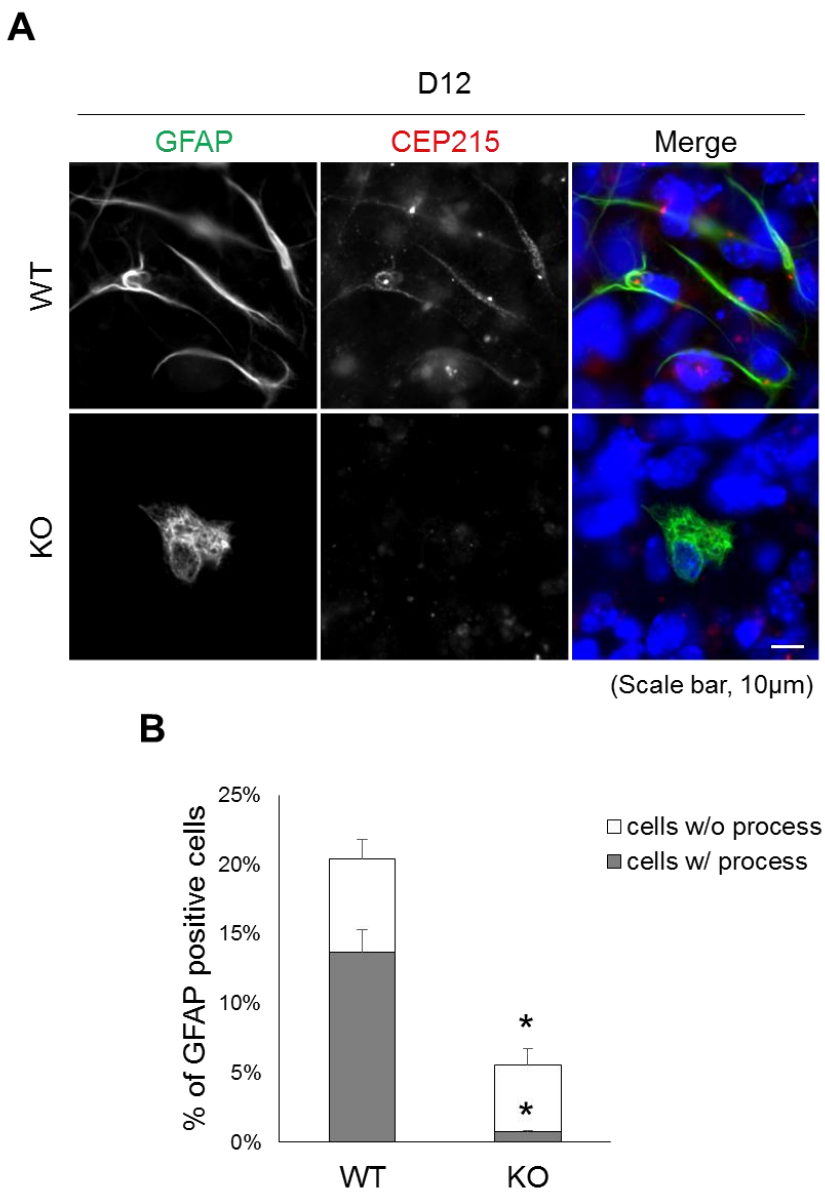


Figure 29. Glial differentiation of the *CEP215*-deleted P19 cells. (A) Wild type (WT) and *CEP215*-deleted (KO) P19 cells at D12 were coimmunostained with the GFAP (green) and CEP215 (red) antibodies. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) The number of GFAP-positive cells with and without processes were counted. More than 400 cells per experimental group were counted in three independent experiments. Error bars; SEM. *P<0.05.

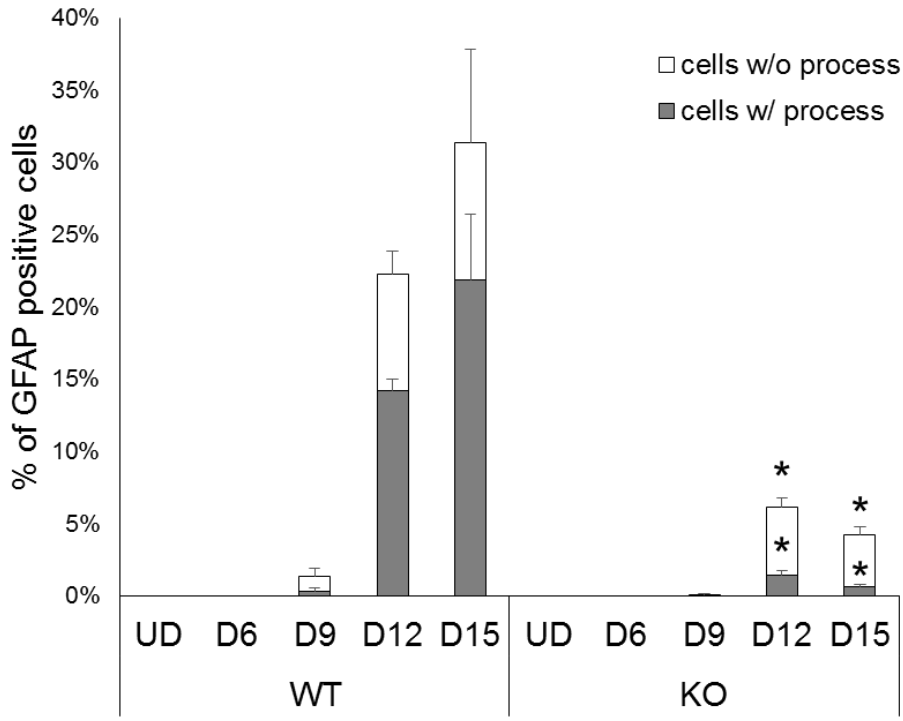


Figure 30. Glial differentiation of the *CEP215*-deleted P19 cells during extended culture periods. Wild type (WT) and *CEP215*-deleted (KO) P19 cells were cultured under a glial differentiation condition. The number of GFAP-positive cells with and without processes were counted at indicated days of differentiated P19 cells. More than 400 cells per experimental group were counted in three independent experiments. Error bars; SEM. *P<0.05.

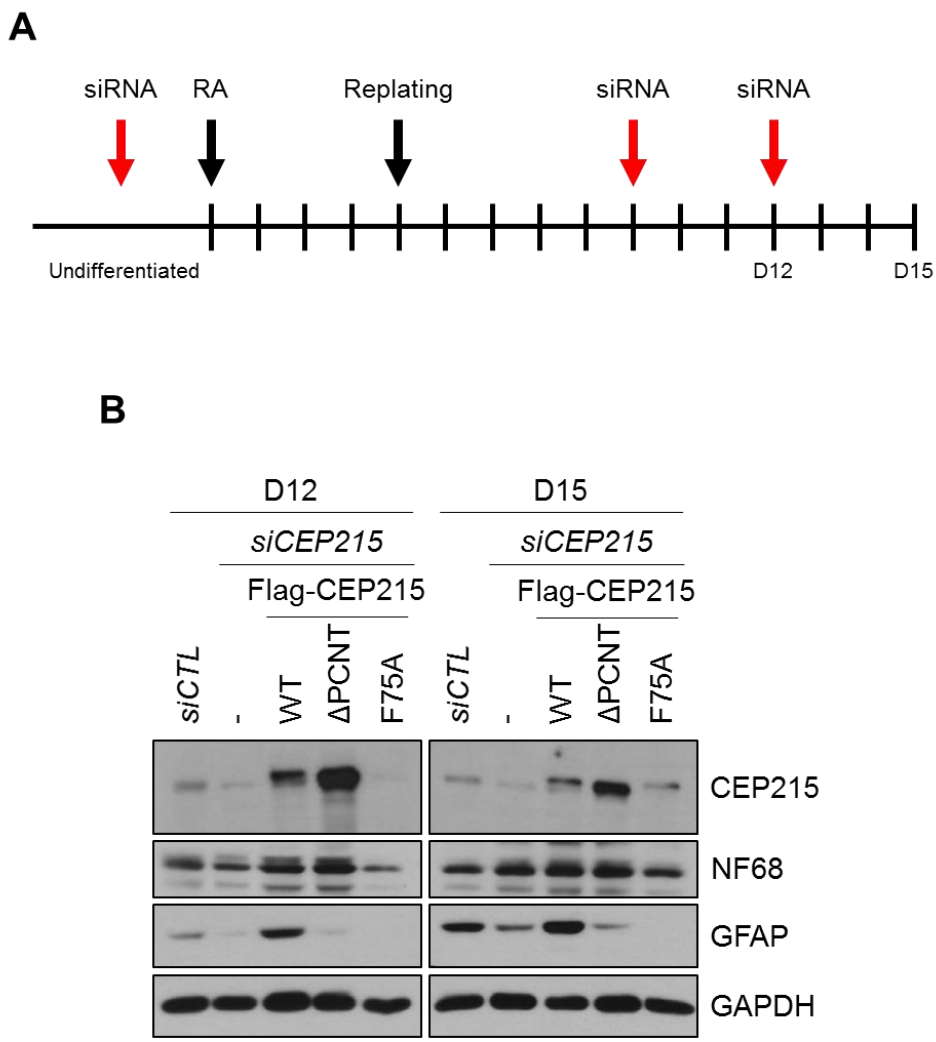


Figure 31. Rescue of the CEP215-depleted P19 cells with ectopic CEP215 constructs. (A) Experimental scheme. P19 cells were stably transfected for expression of Flag-CEP215 (WT), Flag-CEP215$^{\Delta PCNT}$ (ΔPCNT) and Flag-CEP215^{F75A} (F75A). Endogenous CEP215 of P19 cells was depleted with siRNA transfection. (B) Immunoblot analysis of CEP215 was performed at D12 and D15 differentiation stages. NF and GFAP levels were also determined as markers for neuronal and glial cell differentiation, respectively. GAPDH was determined as a control.

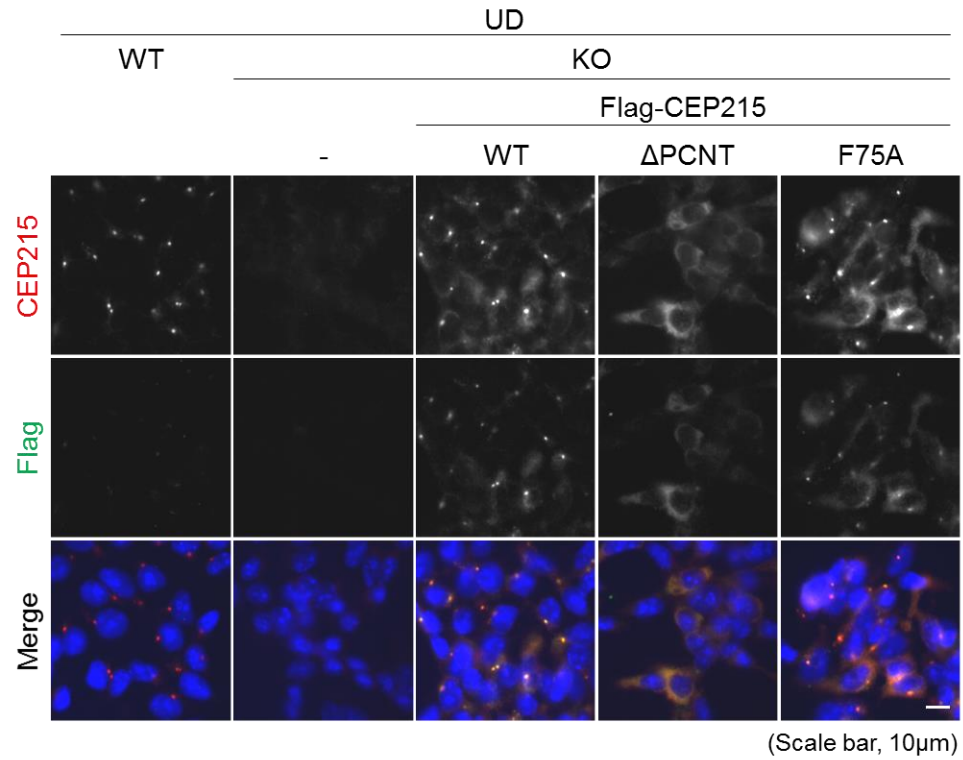


Figure 32. Rescue of the *CEP215*-deleted P19 cells with ectopic *CEP215* constructs in undifferentiated P19 cells. The *CEP215*-deleted P19 cells were stably transfected for expression of Flag-CEP215 (WT), Flag-CEP215$^{\Delta PCNT}$ (ΔPCNT) and Flag-CEP215^{F75A} (F75A). Undifferentiated P19 cells were coimmunostained with the CEP215 (red) and Flag (green) antibodies. Nuclei were stained with DAPI (blue). Scale bar, 10 μm.

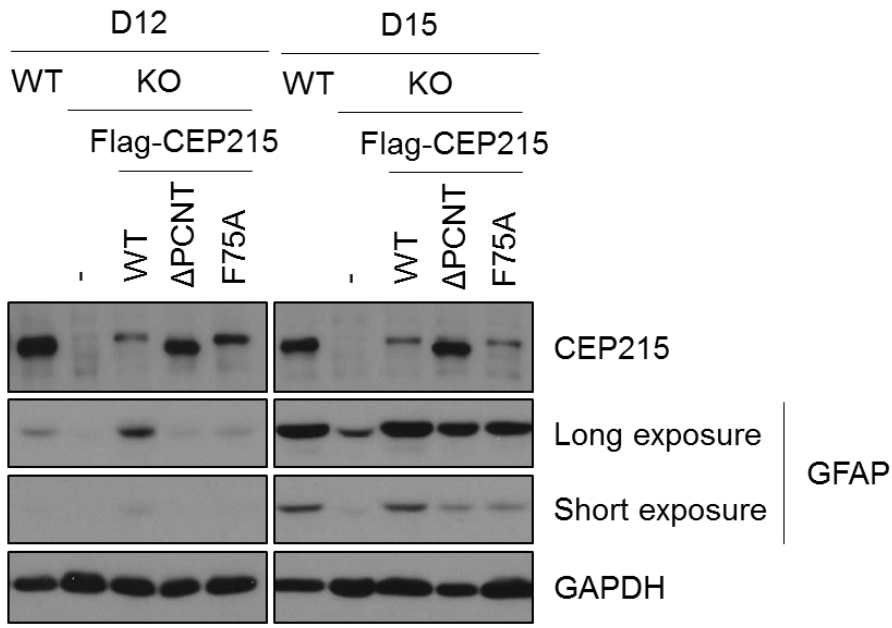


Figure 33. Rescue of the *CEP215*-deleted P19 cells with ectopic *CEP215* constructs.

The *CEP215*-deleted P19 cells were stably transfected for expression of Flag-CEP215 (WT), Flag-CEP215$^{\Delta PCNT}$ (ΔPCNT) and Flag-CEP215^{F75A} (F75A). Cell lysates at D12 and D15 were subjected to immunoblot analysis with antibodies specific to CEP215, GFAP and GAPDH.

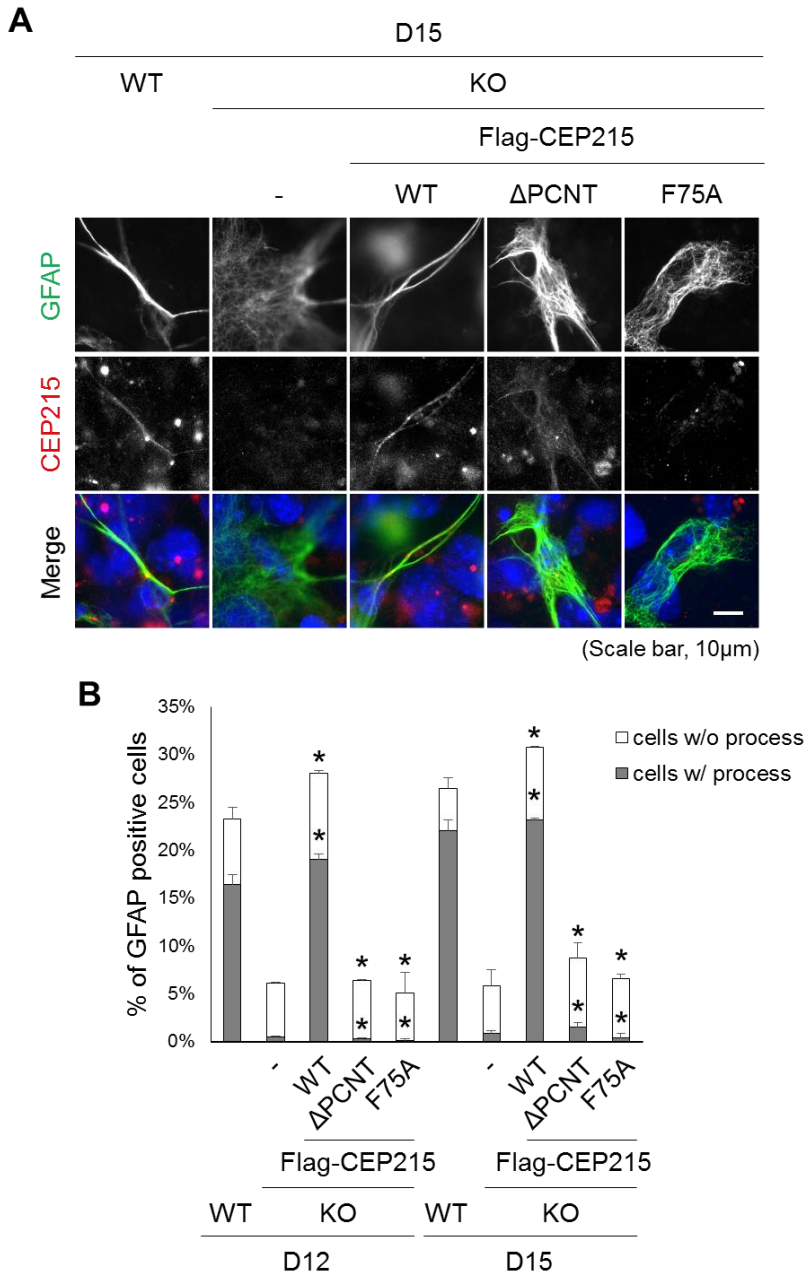


Figure 34. Glial differentiation of *CEP215*-deleted P19 cells rescued with ectopic *CEP215* constructs. (A) The *CEP215*-deleted P19 cells were stably transfected for expression of Flag-CEP215 (WT), Flag-CEP215$^{\Delta PCNT}$ (ΔPCNT) and Flag-CEP215^{F75A} (F75A). Coimmunostaining analyses were performed at D15 differentiation stage with the GFAP (green) and CEP215 (red) antibodies. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) The number of GFAP-positive cells with and without processes were counted at the D12 and D15 differentiation stages. More than 400 cells per experimental group were counted in two independent experiments. Error bars; SEM. *P<0.05.

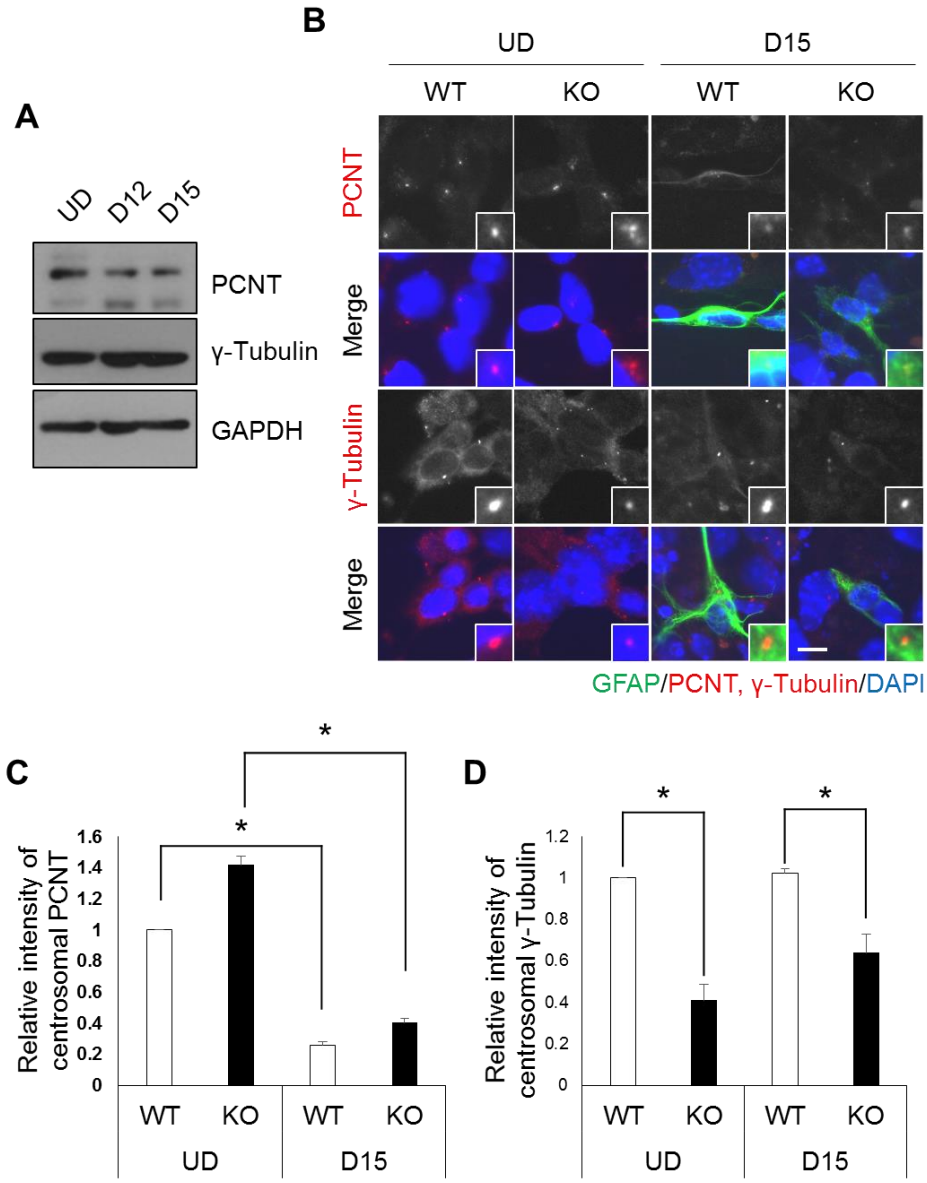


Figure 35. Centrosomal pericentrin and γ-tubulin levels during glial differentiation of P19 cells. (A) Immunoblot was performed to determine the cellular pericentrin (PCNT) and γ-tubulin levels in undifferentiated (UD) and differentiated (D12 and D15) P19 cells. (B) Wild type (WT) and *CEP215*-deleted (KO) P19 cells at UD and D15 were coimmunostained with the GFAP antibody (green) along with the pericentrin or γ-tubulin antibodies (red). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (C) Intensities of the centrosomal pericentrin or γ-tubulin were quantified. More than 30 cells per experimental group were measured in three independent experiments. Error bars; SEM. *P<0.05.

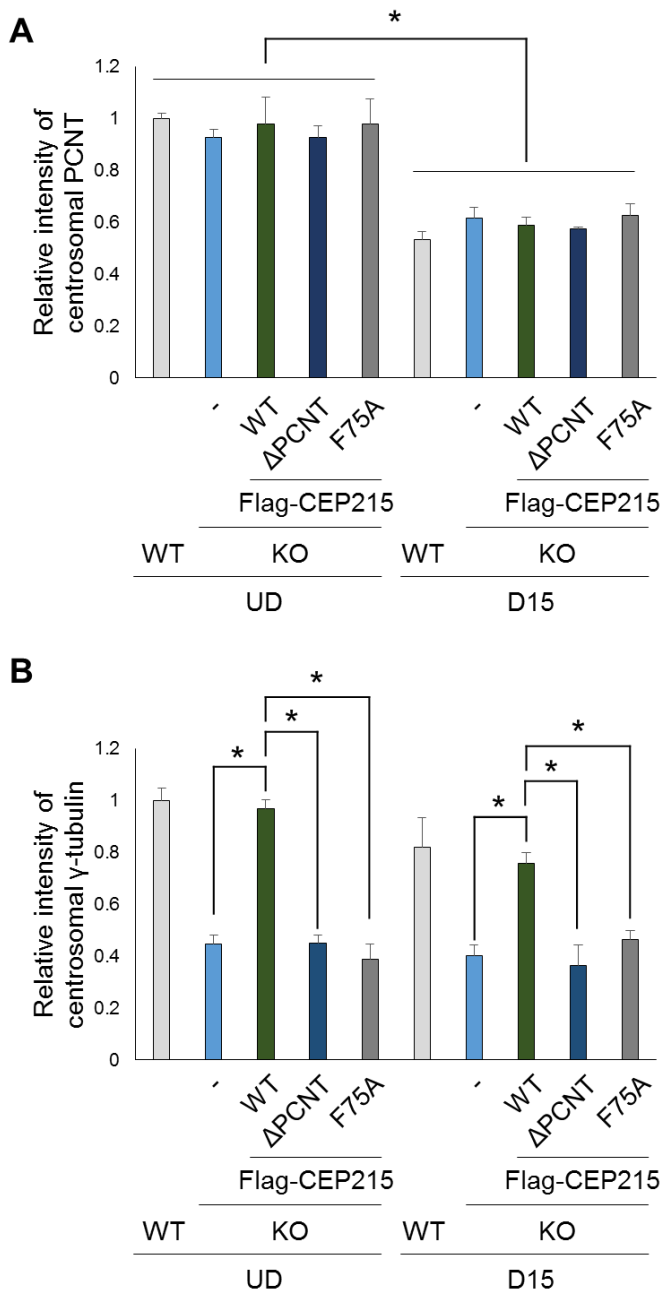


Figure 36. Centrosomal pericentrin and γ-tubulin levels in the *CEP215*-deleted P19 cells rescued with ectopic *CEP215* constructs. The *CEP215*-deleted P19 cells were stably transfected for expression of Flag-CEP215 (WT), Flag-CEP215$^{\Delta PCNT}$ (ΔPCNT) and Flag-CEP215^{F75A} (F75A). Intensities of centrosomal (A) pericentrin and (B) γ-tubulin were quantified at undifferentiated (UD) and differentiated (D15) stages. More than 30 cells per experimental group were measured in two independent experiments. Error bars; SEM. *P<0.05.

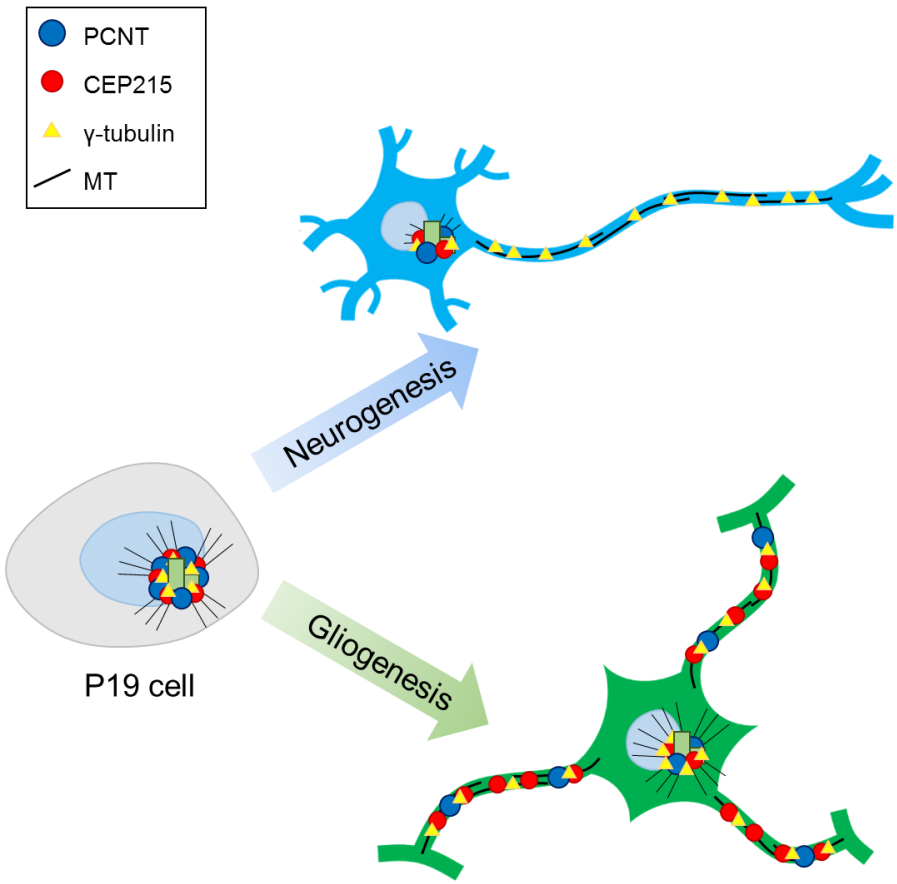


Figure 37. Model. P19 cells may be differentiated into either neurons or glial cells, depending on culture conditions. Both neurons and glial cells include characteristic branches in their cytoplasm. During neurogenesis, microtubule organizing activity at the centrosome is significantly reduced but γ-TuRC localizes on the axon for stable microtubule formation. During gliogenesis, microtubule organizing activity of the centrosomes remains reasonably active. A number of PCM proteins are also localized at the processes of differentiated glial cells. Therefore, processes in glial cells may be an extent of microtubule organization which is operated in the centrosome.

Discussion

In this study, I detected a specific localization of CEP215 along the processes of astrocytes. This expression pattern of CEP215 implies that CEP215 can possibly regulate morphology of astrocytes. The depletion of CEP215 using both siRNA and CRISPR-Cas9 system severely suppressed expression of GFAP in P19 embryonic carcinoma cells, suggesting that CEP215 is essential for glial differentiation. In addition, even GFAP-positive astrocytes in *CEP215*-deleted P19 cells could not organize processes. Meanwhile, other PCM proteins such as pericentrin and γ-tubulin were also detected along the processes of astrocytes. Interestingly, the interaction of PCM proteins were necessary for both glial differentiation and process formation of P19 cells. It was observed that centrosomal γ-tubulin significantly reduced in *CEP215*-deleted cells defected in glial differentiation. These results imply that microtubule nucleating activity of CEP215 is important for glial differentiation and process formation. This is the first study investigated function of centrosomal proteins during gliogenesis.

The range CEP215 caused problem during differentiation of P19 cell must be more closely investigated. In this study, defect of glial differentiation was determined by just using GFAP which is astrocyte specific intermediate filament. Therefore, whether CEP215 affected just expression of GFAP or entire astrocyte differentiation must be further studied. The vimentin which is another astrocyte specific intermediate filament and S100β which is calcium binding protein can be candidates for other astrocyte markers. It was seemed that neuron differentiation confirmed using neuron specific cytoskeleton such as neurofilament and Tuj1 were less affected by *CEP215*

deletion. However, there is a need for study neuronal morphology such as formation of axon and dendrites in *CEP215*-deleted cells more closely. It is also necessary to investigate defects of other types of glial cells such as oligodendrocytes and microglia.

The function of cytoplasmic CEP215 and other PCM proteins is not clear yet. In early neurogenesis the microtubule is nucleated from the centrosome (Stiess et al., 2010). However, the centrosome lose its function as MTOC in mature neurons because centrosomal PCM proteins are depleted from the centrosome (Stiess et al., 2010). In mature neurons, the cytoplasmic γ-tubulin is comparatively increased to maintain the microtubule nucleating activity in the axon, and it is still important for microtubule organization and neurite formation (Sánchez-Huertas et al., 2016). In the case of dendrites in neurons, noncentrosomal microtubule nucleation mediated by Golgi fragments induces dendrite formation. In *Drosophila* dendritic arborization (da) neurons, one of the PCM protein AKAP450 localizes at Golgi fragments, and recruits γ-tubulin (Ori-McKenney et al., 2012). Then, AKAP450 at the Golgi mediates the noncentrosomal microtubule nucleation by γ-tubulin to elongate dendrites in neurons (Ori-McKenney et al., 2012).

The mechanism of process formation in astrocytes is not clear. The microtubule nucleation mediated by γ-tubulin was seemed to be important for process formation of astrocytes, and it was CEP215 dependent. It can be also expected that cytoplasmic CEP215 may recruit γ-tubulin for process formation. It is already known that CEP215 can mediate noncentrosomal microtubule nucleation through its direct binding to γ-tubulin (Choi et al., 2010). It is reported that CEP215 also localizes at Golgi with AKAP450 in somatic cells, and mediates noncentrosomal microtubule nucleation

(Wang et al., 2010; Wu et al., 2016). Therefore, strong cytoplasmic CEP215 implies that the function of AKAP450 during neuronal dendrite formation may be replaced to CEP215 in astrocytes. As a result, PCM protein complex consists of CEP215, pericentrin and γ-tubulin recruits γ-TuRC components to processes of astrocytes, and their microtubule nucleating activity may be important for glial differentiation and process formation of astrocytes (Figure 37).

It must be investigated how the expression of intermediate filament GFAP is regulated by microtubule-associated centrosomal components. It was reported that the other astrocyte specific intermediate filament vimentin is nucleated from centrosome (Trevor et al., 1995). From immunocytochemistry results in mouse hippocampal cells, I also recognized that GFAP was always concentrated around the centrosome like microtubule in both young and mature astrocytes. It was also reported that the organization of intermediate filaments such as vimentin and GFAP depends on microtubule network (Sakamoto et al., 2013). Moreover, microtubule associated motor proteins such as kinesin and dynein also drive polarization of intermediate filament network (Leduc and Etienne-Manneville, 2017). Therefore, it can be assumed that intermediate filament network is affected by centrosomal proteins related to microtubule nucleation. These are first results to show the relationship between centrosomal proteins and intermediate filaments during glial differentiation.

Finally, it was seemed that cell differentiation was determined by centrosomal protein CEP215 which regulate microtubule nucleation. It must be studied why gliogenesis is failed in *CEP215*-deleted cells with defected microtubule nucleation. During progress of neurogenesis, centrosomal proteins can considerably affect neuronal

differentiation and axon formation. During brain development, neuronal precursor cells undergo asymmetric division into neuronal precursor cell and differentiated neuronal cell. The asymmetric division can be maintained by spindle orientation which is decided by centrosomal proteins at the mitotic spindle pole. Considerably, microcephaly is induced by mutants in centrosomal proteins, and following failure of asymmetric division and consequential premature neuronal differentiation (Buchman et al., 2010). Interestingly, CEP215 is the representative microcephaly gene, and interaction of CEP215 and pericentrin is important for asymmetric division of neural progenitor cells (Buchman et al., 2010). In asymmetric neurons, axon specification is also determined by the localization of centrosome (Sakakibara et al., 2013). Taken together, centrosomal proteins can reugulate the cell fate and morphogenesis during neuron differentiaiton. How CEP215 and other PCM proteins regulated the glial differentnaiton must be investigated.

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국문초록

미세소관은 세포의 형태와 세포 내 운송에 관여하는 세포골격이다. 유사분열기 세포에서 미세소관은 딸 세포로 염색체를 전달하기 위한 방추사가 된다. 중심체는 대부분의 동물 세포에서 주요 미세소관 형성 기관으로 기능한다. 하지만 신경 세포나 극성이 있는 표피 세포와 같이 일부 특화된 세포에서는 중심체 외부에서의 미세소관 형성이 관찰된다. 미세소관 형성에 있어서는 γ-tubulin 고리 복합체(γ-TuRC)가 핵심 구조를 담당한다. 따라서 γ-TuRC 복합체의 세포 내 위치와 활성은 세포의 생리적 상황에 맞게 조절되어야 한다. 이 연구에서는 중심체 외부에서 미세소관 형성에 관여하는 centrobin과 CEP215 두 개의 중심체 단백질에 대해 살펴보았다.

제 1장에서는 안정한 미세소관과 연관되어 있는 세포질 특이적 centrobin을 관찰하였다. Centrobin은 중심립 복제에 중요한 딸 중심립 특이적 단백질이다. 해마 세포에서 centrobin은 딸 중심립뿐만 아니라 모 중심립 및 세포질에서도 관찰되었다. 이러한 centrobin의 특이적 위치는 세포질 centrobin이 단순히 중심체로부터 유래된 것이 아니라 특이적 역할을 할 것이라는 것을 보여준다. 실제로 centrobin은 중심체에서뿐만 아니라 외부에서 미세소관 형성의 기능을 보였다. 이러한 결과는 특정 세포 및 세포 주기 동안 세포질 centrobin이 중심체 외부에서 특이적으로 미세소관 형성 기능을 할 것을 시사한다.

제 2장에서는 배양한 생쥐의 해마 세포와 배아 암종 세포인 분화한 P19 세포주에서 성상세포 프로세스 특이적 CEP215의 발현을 관찰하였다.

CEP215가 제거된 P19 세포에서는 GFAP의 발현과 프로세스 형성이 억제되었다. 이러한 CEP215 제거 표현형은 이소성 Flag-CEP215에 의해 회복된 반면, 각각 pericentrin 및 γ-tubulin과 상호작용하지 못하는 Flag-CEP215$^{\Delta PCNT}$와 Flag-CEP215^{F75A} 에서는 회복되지 않았다. CEP215가 제거된 P19 세포에서는 중심체 γ-tubulin의 감소 또한 관찰되었다. 이러한 결과를 바탕으로, CEP215의 미세소관 형성 기능이 교세포 발생에 필수적이라는 것을 알 수 있다.

종합적으로 위의 결과는 centrobin과 CEP215의 중심체 외부에서의 미세소관 형성 기능의 중요성을 보여준다. 이 연구는 교세포 발생 및 성상세포 프로세스 형성에 있어 중심체 단백질의 기능을 최초로 보고하는 바이다.

주요어: 미세소관 핵형성, centrobin, NEK2, CEP215, 교세포발생, 성상세포 프로세스 형성, 중심구

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